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# The bacterial degradation of leucine

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# THE BACTERIAL DEGRADATION OF LEUCINE

by

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# INTRODUCTION

The importance of the central role played by the fixation of carbon dioxide in every branch of the field of biological chemistry is now commonly and universally accepted. Such recognition stems from the historic observations in these laboratories of Werkman and Wood nearly thirty years ago. Since that time one of the primary research interests of this department has been the investigation of the role played by carbon dioxide in the metabolism of a wide variety of microorganisms, both heterotrophic and autotrophic.

The metabolism of the amino acid leucine in mammalian tissues is characterized by a unique carbon dioxide fixation reaction. It was of interest, therefore, to determine whether this same carbon dioxide fixation reaction could be demonstrated in a typical bacterial species during its metabolism of leucine.

An examination of the literature of leucine metabolism reveals a paucity of information concerning the degradation of the amino acid by microorganisms. A number of preliminary studies have been made, but these have not explained the complete utilization or the fate of the carbon skeleton.

For these two reasons, then, the present investigations were undertaken.

# HISTORICAL

It has been known for many years that leucine is an "essential" or "indispensable" amino acid (68), i.e. it must be furnished preformed in the diet of man and higher animals in order to maintain growth and nitrogen balance. The young rat, however, can replace its leucine requirement with α-ketoisocaproic acid; it must therefore be able to bring about an amination of this analog of leucine to synthesize the amino acid. No other precursors have been found which can dispense with the requirement for leucine or α-ketoisocaproic acid in higher animals, however.

The requirement for leucine varies widely in microorganisms. Some bacteria, for example many members of the genera Streptococcus (74) and Lactobacillus (44), require specifically the L-isomer, whereas others can utilize the D-isomer or can even synthesize the entire amino acid from simple precursors, for example Escherichia coli.

The biosynthesis of leucine has been studied in several microorganisms, for example yeasts (Saccharomyces cerevisiae and Torulopsis utilis) (64, 86), bacteria (E. coli) (2, 3) and molds (Neurospora crassa) (2, 3, 6).

Several different methods of approach have been used in these studies. Mutant strains, which have been isolated as the result of genetic damage by such agents as ultraviolet

irradiation, nitrogen mustard, antibiotics, etc., were shown to accumulate certain intermediate compounds in their growth media because of the lack of particular enzymes which catalyzed the chemical transformations of these compounds. From the analysis of these compounds and the subsequent enzymatic demonstrations of their conversions by other organisms, the steps in the metabolic pathway were gradually deduced.

Both the direct and the indirect isotope methods have also been used successfully (64, 86, 2, 3). Labelled precursors, such as $C^{14}$-acetate, -glucose or -lactate, have been supplied to the growing organism in the direct method. After intervals of incubation the distribution of the isotope in cellular constituents was determined by analysis and thus used to deduce the course of synthesis. The indirect, or "isotope competition" method, depends on the ability of an unlabelled compound to decrease the extent of incorporation of an isotope supplied as, say, $C^{14}$-glucose, into cellular components, the unlabelled compound then being considered a normal intermediate in the biosynthesis of the cellular component under investigation.

The studies of the biosynthesis of leucine using all three of these methods have yielded results which are in general agreement. The results of such studies are summarized in Fig. 1.

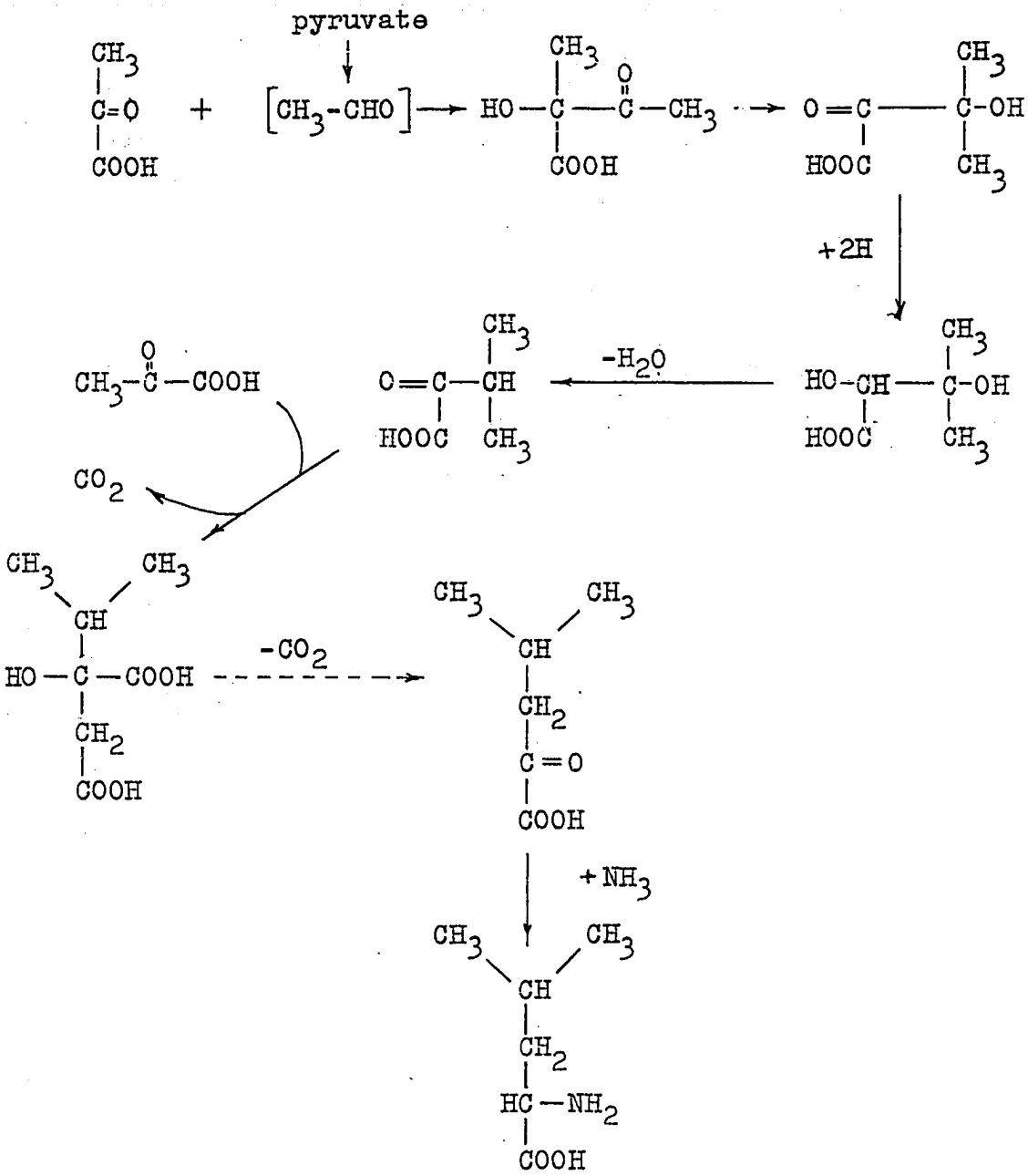


Fig. 1. Microbiological synthesis of leucine

All of the six carbon atoms of leucine have been shown to arise either directly, as in the case of the α- and β-carbons, or indirectly from pyruvate. Whatever the carbon source used initially, glucose, acetate or lactate, it is converted first to pyruvate. Pyruvate condenses with "acetaldehyde", derived from pyruvate, to form α-acetolactic acid (82, 84). This compound then undergoes an intramolecular rearrangement to form α-keto-β-hydroxyisovaleric acid, which is then reduced to the dihydroxy acid. A slightly different scheme for the formation of the dihydroxy acid by Neurospora has been proposed (6). Whatever the actual mechanism, however, the net result is the same. The dihydroxy acid is then dehydrated to form α-ketoisovaleric acid, the keto analog of valine.

α-Ketoisovaleric acid then condenses with "acetate", derived from a third molecule of pyruvate, in a manner closely analogous to the condensation reaction between acetyl-CoA and oxalacetate in the formation of citrate, (85, 86, 64). The seven-carbon compound thus formed is subjected to a series of reactions in close analogy to the conversion of citrate to α-ketoglutarate. The resulting α-keto-isocaproate is converted to leucine by transamination, probably with glutamic acid.

Not all of the above postulated reactions have been demonstrated enzymatically, however.

Little is known about the degradation of leucine by microorganisms (40, p. 787). A number of workers have studied the metabolism of this compound using several types of microorganisms, but no information is yet available concerning the complete utilization of the carbon skeleton.

It has long been known that the "fusel oil" of alcoholic fermentations by yeasts is actually isoamyl alcohol, formed by degradation of leucine, but the mechanism of its formation was not understood (33, 34). This problem has been reinvestigated recently (76, 77) and the deamination and decarboxylation reactions to account for isoamyl alcohol production have been demonstrated. It has been found in the case of S. cerevisiae, using $C^{14}$-leucine, that the carbon skeleton of the amino acid is not appreciably utilized for the synthesis of other amino acids (77).

Enzyme preparations from freeze-dried cells of Proteus vulgaris have been shown to produce isoamyl amine by a decarboxylation of leucine (35). This leucine decarboxylase has been shown to possess properties which are markedly different from the bacterial decarboxylases for other amino acids. Sonic extracts of P. vulgaris also contain an enzyme capable of catalyzing the oxidative deamination of leucine to accumulate $\alpha$-ketoisocaproic acid (87). Clostridium acetobutylicum, on the other hand, accumulates $\alpha$-hydroxyisocaproic acid (40, p. 787).

In a series of studies using Streptococcus lactis var. maltigenes, MacLeod and Morgan have shown leucine to be transaminated and decarboxylated by enzymes present in acetone powders (54). These workers were also able to detect the accumulation of isovaleraldehyde from leucine.

The formation of carotenes by the mold Phycomyces blakesleeanus from leucine labelled in various positions with $C^{14}$ has been investigated by Chichester and his co-workers. The formation of an intermediate $C_2$ compound has been postulated (98, 23).

The degradation of leucine in mammalian tissues (pig heart and rat liver) is now well understood due to the work of Coon and his collaborators. This sequence of reactions is shown in Fig. 2. Both isotopic and enzymatic investigations have contributed to our understanding of this metabolic pathway.

The ketogenic properties of leucine have long been recognized (100) and are now known to be due to the formation of acetoacetic acid (27). Acetoacetate can be formed in two ways: by the condensation of $C_2$ fragments (derived from the $\alpha$- and $\beta$-carbon atoms of leucine), split off in a manner analogous to the oxidation of fatty acids, and by the fixation of $CO_2$ into the isopropyl residue of leucine to form the carboxyl group of acetoacetate. This sequence of

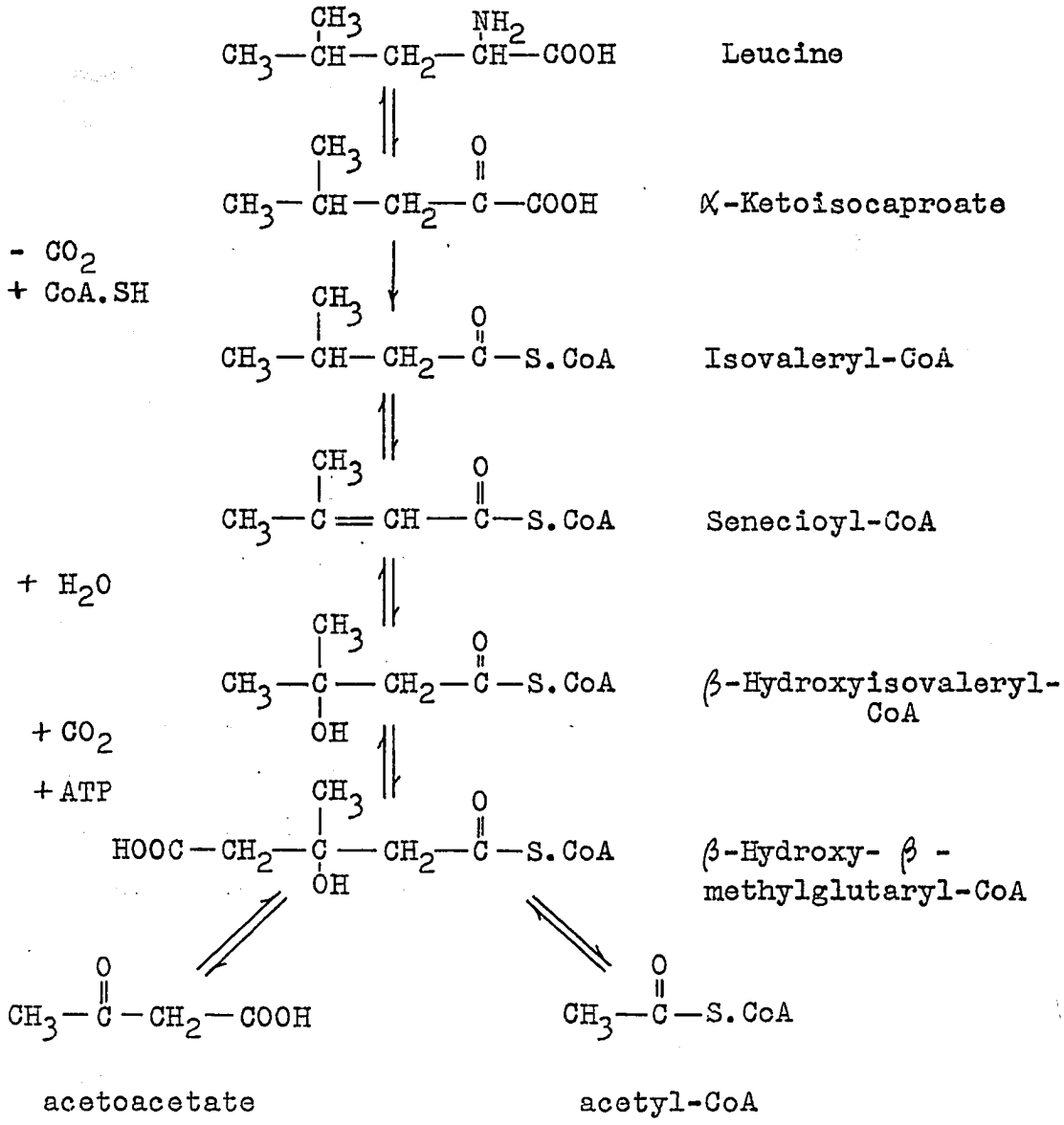


Fig. 2. The mammalian degradation of leucine

reactions involves the formation of intermediate compounds which are esters of Coenzyme A.

α-Ketoisocaproic acid, formed by transamination, is oxidatively decarboxylated to yield isovaleryl-CoA. This reaction, which is irreversible, accounts for the inability of higher animals to synthesize leucine. The oxidative decarboxylation requires diphosphopyridine nucleotide, lipoic acid and thiamine pyrophosphate, and is exactly analogous to the oxidative decarboxylations of other keto acids (e.g. pyruvate and α-ketoglutarate) (42). Isovaleryl-CoA, like the fatty acids, is then oxidized, hydrated and finally condensed with $CO_2$ to form β-hydroxy-β-methylglutaryl-CoA (28, 62). The latter compound is cleaved specifically to yield acetoacetate and acetyl-CoA (66). The detailed investigations of the $CO_2$ fixation step in mammalian leucine degradation have done much to aid in the understanding of the general mechanisms of $CO_2$ fixation.

# MATERIALS AND METHODS

## Organism and Medium

The organism used throughout the experiments reported here was Brevibacterium leucinophagum, a new species isolated from soil (47). A type culture has been deposited in the American Type Culture Collection, Washington, D.C., as well as in the culture collection of the Department of Bacteriology, Iowa State University. In the latter collection it has been given the strain number "14 A 2."

### Isolation

The organism was originally isolated from a sample of flower bed soil on the campus of Iowa State University. A few milligrams of a rich, black top soil were introduced directly into a medium composed of 1% L-leucine and mineral salts adjusted to pH 7.0. After incubation 24 hrs. at 37° C. the heavily turbid broth was streaked on Petri plates containing the same medium solified by the addition of 2% agar. After 24 to 36 hrs. incubation at 37° C. well isolated colonies had developed, which were then picked and transferred to slants of plain nutrient agar.

Stock cultures of the organism were maintained by monthly or bimonthly transfer on plain nutrient agar slants and stored at 4° C. Stock cultures so maintained remained

viable and grew vigorously with minimal lag periods immediately upon transfer into suitable fresh growth media.

Characterization

Standard bacteriological technics were employed in the diagnosis of the species (75). Each of the tests required for conclusive identification was repeated at least three or four times at intervals of approximately two months. The results of all diagnostic tests were consistent, indicating that the original isolate was genetically stable.

Determination of fermentable carbohydrates was made by inoculation of tubes of plain nutrient broth supplemented with 0.5 to 1.0% of the carbohydrate under test with one drop of a washed cell suspension grown 24 hrs. at 30° C. in nutrient broth. The fermentation media contained an inverted vial (Durham tube) and a suitable acid-base indicator, such as bromthymol blue.

## Enzyme Preparations

Whole cell suspensions

Crops of cells for leucine degradation experiments were grown routinely in a medium composed of mineral salts and 0.5% "Sta-mino," type B, a purified soy protein hydrolyzate containing *ca.* 75% L-leucine, manufactured by A. E. Staley Mfg. Co., Decatur, Illinois. When it had been determined

that the species was obligately aerobic, cultures were aerated to increase cell crop yields by swirling on a rotary shaker at 30° C. Growth curve determinations showed that cells grown in this manner were just approaching the end of the logarithmic growth phase after about 30 hrs. Care was therefore taken that all cell crops were harvested during this phase of growth to minimize variation in enzyme content of the cells.

The cells were washed once with distilled water on the centrifuge, then shaken at 30° C. for 2 hrs. in a 1% L-leucine solution to reduce endogenous concentrations of intermediates from the degradation of other growth medium amino acids. The cells were again washed with water, and suspended in water to a concentration of 500 mg. wet weight/ml.

Acetone-dried cells

Washed cell pastes were suspended in distilled water to give a suspension the consistency of a thick cream. This was then added dropwise with vigorous mechanical stirring to 10 volumes of acetone previously chilled to -20° C. Stirring was continued for 10 min., then the cells were allowed to settle as a flocculant precipitate, filtered, and sucked to dryness on a Buchner funnel. Drying was completed over anhydrous $Na_2SO_4$ at -5° C. overnight.

For use in reaction mixtures the acetone powders were suspended in water to give the equivalent of 500 mg. wet cells/ml.

Cell-free extracts

Crude cell-free extracts were prepared by sonic treatment 15 min. in a 9 kc. Raytheon sonic oscillator. Microscopic examination showed ca. 40% cell rupture. Cell debris and unbroken cells were removed by centrifugation at 24,500 x g for 10 min. at 4° C. Extracts thus prepared contained 2.5 to 3.5 mg. protein/ml.

Dialyzed cell extracts

Cell extracts were freed of low molecular weight substances by dialysis in cellophane dialysis bags suspended in a mechanically agitated bath of water and crushed ice.

Dialyzed and undialyzed cell-free extracts were stored at -20° C. if not used immediately.

## Chemicals and Reagents

All chemicals and reagents were obtained from commercial sources and were of reagent grade or comparable purity.

$BaC^{14}O_3$ was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee. This was used to prepare $KHC^{14}O_3$, the source of $C^{14}O_2$ in fixation experiments. $C^{14}O_2$ was liberated from $BaC^{14}O_3$ by 10% $HClO_4$ and absorbed in a solution of KOH

under vacuum. This was then diluted with $CO_2$-free water to a concentration of 0.05 N.

Sodium fluoroacetate (99% pure) was obtained from the University of Chicago Toxicity Laboratory, 927 East 57th Street, Chicago 37, Illinois.

$C^{14}$-L-leucine (uniformly labelled) was obtained from Schwartz Laboratories and possessed a specific activity of 1.15 mc./mg.

DL-leucine-2-$C^{14}$ (specific activity = 0.51 μC/μM) was obtained from the Volk Radiochemical Co.

## Analytical Procedures

### Spectrophotometry

Growth curve determinations were made by reading the transmittance of culture samples in a "Spectronic 20" colorimeter against a blank of sterile medium at a wavelength of 525 mμ. Optically matched, scratch-free test tubes were employed as cuvettes.

### Manometric technics

Standard manometric technics (91) were used for determination of oxygen uptake and carbon dioxide evolution. Both direct and indirect methods for carbon dioxide (91, p. 28) were evaluated. Good agreement of results was obtained by both methods. It was found experimentally that

application of a correction factor for $CO_2$ retention was not required when Tris(hydroxymethyl)aminomethane (tris) buffers were used in place of phosphate buffers.

Measurement of radioactivity

Radioactivity of reaction mixture was measured by drying 50 or 100 µl. aliquots quickly on etched glass planchets under a gentle blast of warm air from a hair dryer. The temperature was not allowed to exceed 55° C. Samples were counted under an end-window GM tube, the geometry of which gave an overall counting efficiency of ca. 10%. No corrections were made other than background since the same standardized methods were employed in all experiments; this was considered sufficient for comparative purposes.

Chromatography

Chambers and papers Wide-mouth gallon jars with screw-capped lids were used for ascending paper chromatography. Commercially-obtained rectangular chambers fitted with plate glass lids and stainless steel troughs were used for descending technics. Whatman no. 1 paper was used routinely throughout except when large scale separations were required. In this case Whatman no. 3MM paper was used since larger sample aliquots could be applied without fear of "tailing." To obtain complete separations in some cases

it was necessary to remove residual impurities from the papers by washing in 0.1 M citric or oxalic acids (18 p. 157).

Solvents A variety of solvent systems was used depending on the types of compounds to be separated as well as for purposes of comparison in identification of unknown compounds.

Phenol: A water-saturated solution was prepared, then a trace more phenol was added to prevent separation into phases due to temperature variations.

BAW: The organic phase of the system n-butanol:glacial acetic acid:water (4:1:5, v/v/v) was used. A small beaker containing a portion of the aqueous phase was included in the chamber for saturation of the atmosphere.

EAW: The system contained 95% ethanol:concentrated $NH_4OH$:water (80:5:20, v/v/v).

EAFW: This solvent was made up with ethyl acetate: concentrated formic acid:water (30:10:5, v/v/v).

BEA: This solvent is especially well-suited to the separation of carbonyl compounds as the 2,4-DNP derivatives (18, p. 174), and is composed of n-butanol:95% ethanol:0.5 N $NH_4OH$ (7:1:2, v/v/v).

All chromatograms were developed at room temperatures (ca. 25° C.) except in certain cases employing EAFW. This solvent migrates very rapidly, especially with Whatman no. 1

paper, so that better separations were obtained when development took place at 10° C.

Spraying reagents Colorless compounds were located on the chromatograms by spraying with one of the following reagents:

Ninhydrin: For detection of amino acids and amines a 0.1% solution in 95% ethanol was sprayed on the paper and allowed to dry. The chromatogram was then steamed for a minute or two in the autoclave at 100° C. to develope the color.

Mixed indicators: A mixture of 0.04% bromphenol blue and 0.04% methyl yellow or methyl red in 95% ethanol, adjusted to pH 7.5 with KOH, was used to locate acidic or basic substances.

Nitroprusside reagent: This reagent was useful for detecting substances containing free sulfhydryl groups (89). CoA esters were visualized after first spraying with 10% KOH or NaOH to hydrolyze the thiol ester linkage. Sodium nitroprusside (1.5 g.) was dissolved in 5 ml. 2 N $H_2SO_4$, and then 95 ml. absolute methanol was added, followed by 10 ml. 28% $NH_3$. The thick precipitate was filtered off, and the filtrate, which was stable for two months, was stored at 4° C. Free sulfhydryl groups give a deep violet color when sprayed.

All chromatograms were routinely examined under both ultraviolet and visible light before and after spraying with reagents. No spray was necessary for detection of 2,4-DNP derivatives of carbonyl compounds since the yellow derivatives absorbed strongly under ultraviolet light.

Radioautography

Radioactive compounds were located on paper chromatograms by exposure to Kodak No-Screen X-Ray film for periods of 5 to 10 days, depending on the activity of the samples. Films were developed in Kodak D-16 developer 6 to 10 min. at 25° C., washed briefly in 0.01% acetic acid, then cleared for 10 min. in 5% $Na_2S_2O_3$. The films were washed one hour in running water to remove all traces of thiosulfate.

Chemical methods

Hydroxamic acid derivatives The method of Stadtman and Barker (78) was used with slight modification. The deproteinized samples (2 ml.) were treated with 1 ml. neutral 2 M $NH_2OH$ for 15 min., then extracted with 20 ml. 95% ethanol. The ethanol extract was evaporated on a hot water bath to 3 to 4 ml., then to near dryness at room temperature under a gentle blast of air. Five ml. absolute methanol were added, then evaporated at room temperature to 0.1 to 0.2 ml. This was used for chromatography. Hydroxamates give a deep red color with 0.6% methanolic $FeCl_3$.

In addition to the other solvents listed above, water-saturated n-butanol was occassionally used for chromatographic operation of hydroxamates.

Coenzyme A esters These were identified according to the method of Stadtman (80), and were detected with the nitroprusside reagent of Toennies and Kolb (89) as described above.

Acetoacetate The potassium salt of acetoacetic acid was prepared by alkaline hydrolysis of the ethyl ester according to Ljunggren (52), and estimated colorimetrically by the diazo technic of Walker (93). No modifications of these methods were made.

Amino acids These were estimated quantitatively according to the following procedure. The amino acids were located on paper chromatograms by spraying with 0.01% ninhydrin in 95% ethanol. The spots were excised from the paper, sliced up in a test tube, care being taken to avoid touching the papers with the fingers, and heated 5 min. on a water bath with 2 ml. 0.5% ninhydrin in n-butanol. The colored extracts were diluted to constant volume with 80% acetone and read at 560 mu against a blank prepared from filter paper.

Ammonia This was determined by the standard Nesslerization method, either with or without previous distillation. Samples containing protein or other interfering

compounds were distilled in the presence of alkali in a Parnas apparatus into 0.01 N $H_2SO_4$. The Nesslerized samples were read at 490 mμ against suitably prepared standards.

Carbonyl compounds The 2,4-dinitrophenylhydrazones were prepared as follows. Samples were freed of denatured protein by centrifugation after treatment with trichloracetic acid. To 1 ml. of the sample was added 0.5 to 1.0 ml. 0.5% 2,4-dinitrophenylhydrazine in 6 N HCl. The tubes were incubated 30 min. at 25° C. to allow formation of the hydrazone. The samples were then extracted with three 15 ml. portions of $CHCl_3$ containing 20% ethanol. The combined extracts were extracted then with 15 ml. N/1 $Na_2CO_3$ to remove acidic 2,4-DNP's. The alkaline extract was washed with $CHCl_3$-EtOH. Acetone-2,4-DNP and other neutral derivatives remained in the $CHCl_3$-EtOH fraction, which could then be extracted with $CCl_4$. The alkaline extract containing the acidic 2,4-DNP's was then acidified with 5 ml. 6 N HCl, and re-extracted with 15, 10 and 5 ml. portions of $CHCl_3$-EtOH. These were then evaporated to dryness under a gentle air blast.

Protein This was estimated spectrophotometrically (79) using crystalline bovine serum albumin (Armour) as standard.

## Identification of Unknown Compounds

Unknown compounds in reaction mixtures were identified by paper chromatography tentatively from their $R_f$ values, then excised from the paper, eluted and cochromatographed with authentic compounds in at least two, generally three, solvent systems to confirm their identities. The very small amounts of materials recoverable precluded the use of classical chemical classification and identification procedures.

# EXPERIMENTAL

Since it was desired to study the complete degradation of the carbon skeleton of leucine, it was essential that a test organism be chosen which possessed the enzymatic machinery capable of utilizing the entire leucine molecule. Since it would be a formidable task to survey a large number of known species to find one which met this requirement, it was decided to isolate an organism from Nature on L-leucine as sole carbon and nitrogen source, the logical conclusion being that an organism would thus be selected which possessed the necessary complement of enzymes to degrade completely the leucine molecule.

It was readily observed that the properties of the test organism finally chosen did not correspond with those of the more familiar species found in our laboratories. A detailed study was therefore made which soon proved the isolate to be a species which had not been described previously.

## Characterization of the Organism

### Cultural characteristics

Flasks of liquid nutrient media inoculated with B. leucinophagum showed silkiness when swirled after being incubated 8 to 12 hrs. at 30° C. Turbidity became moderately heavy, and a thin membrane formed on the surface which was

easily dislodged and settled to the bottom of the flask to form a thin sediment while the broth remained turbid. The slight ring which remained at the surface soon gave rise to another membrane.

Colonies grown on the surface of nutrient agar or nutrient gelatin plates were small, generally not more than 2 mm. in diameter, circular with an entire edge, raised, butyrous and glistening. When viewed by reflected light, the colonies were white to buff-colored, but by transmitted light were iridescent. Growth on nutrient agar slants was moderate to heavy and possessed a beaded appearance.

The formation of a membrane on the surface of liquid media indicated a strong affinity for oxygen. It was demonstrated, furthermore, that the species is obligately aerobic, since no growth occurred in deep tubes of gelatin or agar media when stab-inoculated, either in the presence or absence of a fermentable carbohydrate. Similarly, glucose broth tubes overlaid with a layer of sterile mineral oil did not support growth. Crops of cells grown in broth were increased three- or fourfold in yield by vigorous aeration. As will be seen below, no degradation of leucine occurred unless aerobic conditions were present.

Morphological characteristics

The cells were readily and uniformly stained by the usual aniline dyes, and when stained according to the Gram

technic were Gram positive. This species characteristically loses its Gram positive character after 12 to 16 hrs., however, and then resembles very closely a typical Gram negative rod, such as a pseudomonad, with which it was confused, as a matter of fact, on original isolation. The cells are very short, stubby rods, measuring 0.4 to 0.7 by 0.7 to 1.2 microns. Young cells from liquid cultures often resemble cocci or "stretched cocci," but as the culture ages longer rod-shaped forms are often observed. At temperatures above 30° C., the optimum, long, unbranched filaments occur and the cells tend to pleomorphism. No characteristic cell arrangements have been observed. Endospores are not formed, and lipid inclusion bodies are not formed when the species is grown in carbohydrate media. The organism is not motile.

Physiological characteristics

A number of common physiological test media were inoculated to determine the following properties of the species.

Acid, but no gas, is readily produced in glucose broth; methyl red tests are negative, however, and no acetylmethylcarbinol is formed. No acid or gas production was noted when fructose, lactose, sucrose, maltose, mannose, raffinose, glycerol, mannitol, sorbitol and salicin were tested. When first isolated the species produced very slight traces of acidity (as determined by bromthymol blue indicator) but no gas in the presence of xylose, but this property was lost

after a few transfers on nutrient agar and could not be recovered by repeated culture in the presence of xylose. A slightly basic reaction was usually noted in peptone media (pH 7.5 to 7.6 by the glass electrode), but no free ammonia could be detected in growing cultures by the use of Nessler's reagent. Resting cells did produce small amounts of ammonia under some conditions from some substrates, however.

Nitrates are not attacked, nor is urea. Moderate to heavy growth can be obtained in 24 to 36 hrs. at 30° C. when washed cells are inoculated into media containing ammonium phosphate ($NH_4H_2PO_4$) as sole source of nitrogen.

Since B. leucinophagum does not grow anaerobically in stab cultures, gelatin hydrolysis was tested by the Frazier method as modified by Smith (72). Gelatin is not hydrolyzed. Hydrogen sulfide could not be detected when agar plates containing either lead acetate or ferrous sulfate were inoculated with the test organism. Starch is not hydrolyzed.

Indole is not produced in Bacto-tryptone (Difco) broth cultures. Repeated subculture in the chemically defined medium of Koser yields heavy growth, indicating that citrate is readily utilized as sole carbon source. Other intermediates of the tricarboxylic acid cycle ( $\alpha$-ketoglutarate, succinate, malate, fumarate and acetate) are rapidly utilized; the presence and operation of this cycle has been demonstrated (see next page). A number of other substrates (glucose, ethanol,

acetoacetate) are also rapidly oxidized, either by whole cells or, in some cases, by acetone-dried cell suspensions also.

When tubes of litmus milk were incubated at 30° C., no immediate changes took place. After 4 to 5 days, however, the litmus in the depths of the tubes was reduced slowly, and after 10 to 12 days the contents of the tubes became solid. Litmus at the surface remained blue, indicating that the curd which formed was not due to accumulation of acid. No peptonization of the casein was apparent, however, even after 14 days.

No hemolysis occurs on sheep blood agar plates at 30° C. or 37° C.

Cultures of this species remain viable for periods up to 3 months when agar slants are stored aerobically at 4° C.; broth cultures at this temperature do not remain viable longer than 3 to 4 weeks, however. On the other hand, washed cell suspensions retained their ability to oxidize leucine rapidly up to 8 weeks when stored at 4° C.! Cultures grow well, though slowly, at 10° C., and show optimum growth at 30° C. At 37° C. many long, unbranched filaments and pleomorphic forms are produced rapidly in liquid media. No growth occurs at 45° C.

## Preliminary Studies of Leucine Degradation

It was of interest initially to obtain some information on the relative rates of leucine utilization by B. leucinophagum under conditions simulating those found in growing cultures. Consequently small test tubes containing solutions of leucine were heavily inoculated with washed whole cells and incubated at 37° C. Aliqots of the reaction mixtures were withdrawn from replicate tubes at intervals and analyzed for residual leucine by a combination of the chromatographic and colorimetric technics previously described. Measured aliquots were submitted to quantitative separation on paper, the leucine was recovered, and assayed. The results of such an experiment are shown in Table 1.

Table 1. Disappearance of leucine during degradation by washed whole cells[a]

| Time, hrs. | Leucine remaining[b] µM | Leucine utilized µM |
|---|---|---|
| 0 | 11.7 | 0 |
| 1 | 8.9 | 2.8 |
| 2 | 5.2 | 6.5 |
| 3 | 2.3 | 9.4 |
| 4 | 0.8 | 10.9 |

[a]Reaction mixtures contained 100 µM tris, pH 7.5; 10 µM L-leucine (calcd.); 150 mg. wet cells. Total volume 3.0 ml. Incubated at 37° C. under air (stationary). Reaction stopped by the addition of 0.3 ml. 50% trichloracetic acid.

[b]Measured by the ninhydrin colorimetric method.

The leucine appeared to be utilized rather slowly, more slowly than was expected since the rate of disappearance was hardly commensurate with normal growth. A number of other amino acids were found on the chromatograms, apparently leached from the cells by the chromatographic solvents, since aliquots of the whole reaction mixture were chromatographed. To determine whether these slow rates of leucine uptake might be due to the subtle effects of the reaction conditions, interference by other amino acids, or simply spurious assay results, the analytical method was tested for its reliability. Tables 2 and 3 show the efficiency of recovery both directly from a solution of leucine alone, and by chromatography from a mixture of four amino acids.

Table 2. Direct recovery of leucine from solution

| Leucine added (calcd.) µg./10µl. | Leucine recovered µg./10µl. | Error % |
|---|---|---|
| 5.0 | 5.20 | 4.0 |
| 10.0 | 10.4 | 4.0 |
| 20.0 | 19.2 | -4.0 |
| 40.0 | 37.3 | -6.7 |

Table 3. Chromatographic recovery of leucine from a mixture of amino acids

| Leucine added (calcd.) µg./10µl. | Leucine recovered[a] µg./10µl. | Error % |
|---|---|---|
| 5.0 | 4.63 | -7.4 |
| 10.0 | 9.11 | -8.9 |
| 20.0 | 21.3 | 6.3 |
| 40.0 | 36.3 | -9.2 |

[a]Multiple spots were pooled if quantities greater than 10 µg. leucine were required.

Since the assay results were thus within tolerable limits of error (±10%), it must be concluded that these low utilization rates are an artifact of the reaction conditions. This was later confirmed when it was found that higher rates of oxidation, and thus degradation, were possible when conventional Warburg flasks were used as reaction vessels rather than deep test tubes. Flasks were therefore used routinely thereafter.

Manometric studies

Only the L-isomer of leucine is metabolized by B. leucinophagum, as is shown in Table 4. The D-isomer is not

only not oxidizable, but appears to inhibit the rate of oxidation of the L-isomer by whole cells.

Table 4. Oxidation of D-, L- and DL-leucine by whole cells[a]

| Time, min. | µl. Oxygen Uptake | | |
|---|---|---|---|
| | D-leucine | L-leucine | DL-leucine |
| 10 | 0 | 49 | 22 |
| 20 | 1 | 96 | 55 |
| 30 | 3 | 159 | 101 |
| 40 | 8 | 217 | 135 |

[a]Flasks contained 100 µM tris, pH 7.0; 6.25 µM leucine; 150 mg. wet cells. Total volume = 2.0 ml. Temperature = 30.3° C.; gas phase = air. Center well contained a fluted filter paper + 0.1 ml. 20% KOH. Data corrected for endogenous respiration.

Since it is now commonly accepted that all heterotrophic organisms fix $CO_2$, and that this reincorporation of $CO_2$ evolved from a substrate may influence metabolic patterns, it is essential to compare the rates of oxygen uptake both in the presence and absence of $CO_2$ in preliminary studies of a metabolic pathway.

Identical quantities of washed whole cells were used to measure rates of oxygen uptake on leucine by both the direct and the indirect methods, i.e. both in the presence or absence

of $CO_2$ which might be evolved from leucine. This also allowed a measurement of $CO_2$ evolution as a result of possible decarboxylation mechanisms. These results are shown in Table 5.

Table 5. $O_2$ uptake and $CO_2$ evolution on leucine[a]

| Time, min. | Oxygen Uptake µl. | Carbon Dioxide Evolution µl. |
|---|---|---|
| 10 | 50 | 84 |
| 20 | 87 | 144 |
| 30 | 93 | 154 |
| 40 | 116 | 186 |
| 50 | 125 | 199 |
| 60 | 133 | 212 |
| 70 | 137 | 213 |
| 80 | 149 | 227 |

[a]Flasks contained 100 µM tris, pH 7.0; 8.35 µM L-leucine; 150 mg. wet cells. Temperature = 30.3° C; gas phase = air. Results calculated by the indirect method.

The same batch of washed whole cells was used in the experiments represented by the data of Tables 4 and 5. It can be seen that not only are considerable quantities of $CO_2$

evolved from leucine, but that this $CO_2$ influences strikingly the rates of oxygen uptake on similar amounts of substrate. This would suggest that a fixation of $CO_2$ might play a role either directly or indirectly in the metabolism of leucine by this organism.

The ratio of $CO_2$ and $O_2$ exchanged (R.Q..) can often be used to derive valuable information concerning the pathway of metabolism of an oxidizable substrate (91, p. 31). All attempts to obtain this information with this organism were unsuccessful, however. On nine different occassions using different batches of cells (presumably prepared identically) the R.Q. varied from 0.50 to 0.85. Although the R.Q. varied according to the phase of growth of the harvested cells, it was apparently due also to other subtle influences which are more obscure. No stoichiometric relationships could be shown. These determinations in this organism are complicated by the fact that the substrate serves as an adequate source of both carbon and nitrogen, hence in buffered substrate solutions the test cells cannot be maintained in a "resting" state and the metabolic picture is complicated by all the dynamic processes of growth and reproduction.

Deamination

During the early growth studies and classical physiological tests it was observed that the reaction of the medium became slightly alkaline. To determine whether this might be

the result of an active deamination process in the degradation of leucine, reaction mixtures were analyzed for the accumulation of free ammonia.

Again, inconsistent results were obtained due to complications by a number of factors. No free ammonia could be detected in the presence of growing cells, though this was to be expected since $NH_4^+$ serves as a readily assimilable N source. The presence of an active deaminase was indicated, however, by experiments with suspensions of acetone-dried cells (Table 6). The importance of this reaction in leucine degradation cannot be assessed, however, without resort to $N^{15}$ experiments.

Table 6. $NH_3$ formation from leucine by acetone powders[a]

| Time, hrs. | $NH_3$, total µM/ml. | $NH_3$, net µM/ml. |
|---|---|---|
| 0 | 1.7 | 0 |
| 1 | 2.4 | 0.7 |
| 2 | 3.1 | 1.4 |
| 3 | 3.5 | 1.8 |
| 4 | 3.5 | 1.8 |

[a]Reaction mixtures contained 200 µM tris, pH 7.0; 10 µM L-leucine (calcd.); acetone powder equivalent to 700 mg. wet cells. Total volume = 3.00 ml./flask. Incubated at 30° C. under air (stationary). Reaction stopped by addition of 0.2 ml. 50% trichloracetic acid. $NH_3$ measured by direct Nesslerization of deproteinized supernatants.

Transamination

In addition to oxidation, decarboxylation and deamination, a general mechanism of metabolism of amino acids is the participation in transamination reactions.

B. leucinophagum was found to possess a very active transaminase system between leucine and α-ketoglutarate which could be easily demonstrated in both whole and acetone-dried cells and with cell-free extracts. Table 7 represents a typical transaminase balance study employing a crude sonic extract. Since B. leucinophagum readily assimilates $NH_4^+$, and α-ketoglutarate (therefore presumably glutamate also)

Table 7. Transamination between leucine and α-ketoglutarate[a]

| Time, hrs. | Leucine utilized, μM/ml. | Glutamate formed, μM/ml. |
|---|---|---|
| 1.2 | 4.06 | 2.04 |
| 2.0 | 4.57 | 2.47 |
| 3.1 | 5.23 | 2.23 |
| 5.5 | 5.79 | 2.14 |

[a]Reaction mixtures contained 100 μM tris, pH 7.5; 25 μM L-leucine; 25 μM α-ketoglutarate; 20 μg. pyridoxal phosphate; 0.5 ml. crude juice (2.7 mg. protein) in a total volume of 2.60 ml. Incubated at 37° C. under air. Reaction stopped by the addition of 0.3 ml. 50% trichloracetic acid.

participates in transamination, it may be postulated that glutamic dehydrogenase plays a key role in the incorporation of inorganic $NH_4^+$. The differences in the glutamate assay results of Table 7 are significant at the P = 0.10 level. It is of interest that after prolonged incubation, accumulated glutamate is slowly utilized. A discrepancy is apparent between the amounts of leucine disappearing and the amounts of glutamate being formed. Meister (56) has stated that for conclusive demonstration of the occurrence of transamination, a complete stoichiometric balance must be in evidence. On the basis of the fact that glutamate is significantly utilized between times 2.0 and 5.5 hrs., it must be assumed that glutamate-utilizing reactions are occurring which complicate the picture, since the appearance of a carbonyl compound corresponding to α-ketoisocaproate, the keto analog of leucine, supports the evidence for a transamination. No transamination balance studies of shorter than one hour duration were conducted to test this hypothesis, however.

## Degradation of Radioactive Leucine

The information gained through these preliminary degradation studies permitted the postulation of some reactions to be investigated in greater detail. For example, as a result of decarboxylation reactions one might expect detectable accumulations of the appropriate amine, viz.

isoamylamine. All attempts to demonstrate the presence of this compound were unsuccessful, however. Similarly, the expected products of deamination reactions could not be shown. It was therefore decided to use the isotopic approach which would enable one to detect much smaller quantities of any degradation products formed which could not be detected by less sensitive chemical means.

DL-leucine-2-$C^{14}$

Since it had been demonstrated that large quantities of $CO_2$ were evolved on leucine, supposedly the result of a typical amino acid decarboxylation, the negative evidence of isoamylamine accumulation notwithstanding, radioactive leucine labelled in the C-2 position was selected for initial degradation studies. This would allow tracing of the residual carbon skeleton even though the C-1 should be lost through decarboxylation.

Washed whole cells were incubated aerobically in the presence of DL-leucine-2-$C^{14}$ in conventional Warburg flasks containing a fluted filter paper in the center well with 0.1 ml. 20% KOH to absorb evolved $CO_2$. After incubation for variable times at 30.3° C. the reactions were stopped by immediate freezing of the reaction mixtures at -20° C. until subsequent chromatographic analysis. As a matter of routine, the filter papers were examined after completion of the

reactions, and it was discovered that large amounts of $C^{14}O_2$ (from the C-2 position) had unexpectedly been absorbed. Very crude measurements of this radioactivity could be made by pressing the KOH papers onto greased planchets and counting under a GM tube (Table 8). Considering the fact that the overall efficiency of such a crude detection method must be very low (5% or less), it is apparent that the measurement of the wet KOH papers (which rapidly lost their activities as they dried) represents a significant degradation involving the C-2 position.

Table 8. $C^{14}O_2$ from DL-leucine -2-$C^{14}$ [a]

| Time, min. | Counts per min. [b] |
|---|---|
| 30 | 1912 |
| 60 | 1874 |
| 120 | 1798 |
| 180 | 1870 |

[a] Degradation mixtures contained 100 μM tris, pH 7.0; 5 μM DL-leucine-2-$C^{14}$ (ca. $5.6 \times 10^6$ disintegrations/min., calcd. from the specific activity); and 300 mg. washed cells in a total volume of 2.0 ml. Incubated at 30.3° C. under air. Filter papers containing KOH were placed in the center well.

[b] Averages of three 1-min. counts made on each paper in quick succession.

The samples from the above experiment were analyzed chromatographically and radioautograms prepared to detect radioactive compounds. The results are shown in Fig. 3. The spots were very faint and difficult to detect due to their low specific activities. A number of ninhydrin-positive areas were detected on the chromatograms after spraying, some of which corresponded with the faintly radioactive spots.

It was apparent that the very low amounts of radioactivity retained in the degradation products would render recovery and subsequent identification procedures very difficult. The majority of radioactivity was lost as $CO_2$, the small amounts remaining representing perhaps alternate pathways of degradation of leucine or metabolism of some intermediate $C_2$ fragment before it was finally converted to $CO_2$.

To determine whether leucine might be degraded anaerobically to yield different products, or a least products containing higher concentrations of radioisotope, the degradation experiment was repeated under an atmosphere of 100% $N_2$, provided by flushing the reaction vessels at least 15 min. before the experiment was begun with tank $N_2$ from which traces of $O_2$ had been removed by passing over hot Cu turnings. No degradation of the leucine occurred under these conditions: no radioactive spots other than leucine were detectable, and no $C^{14}O_2$ was evolved.

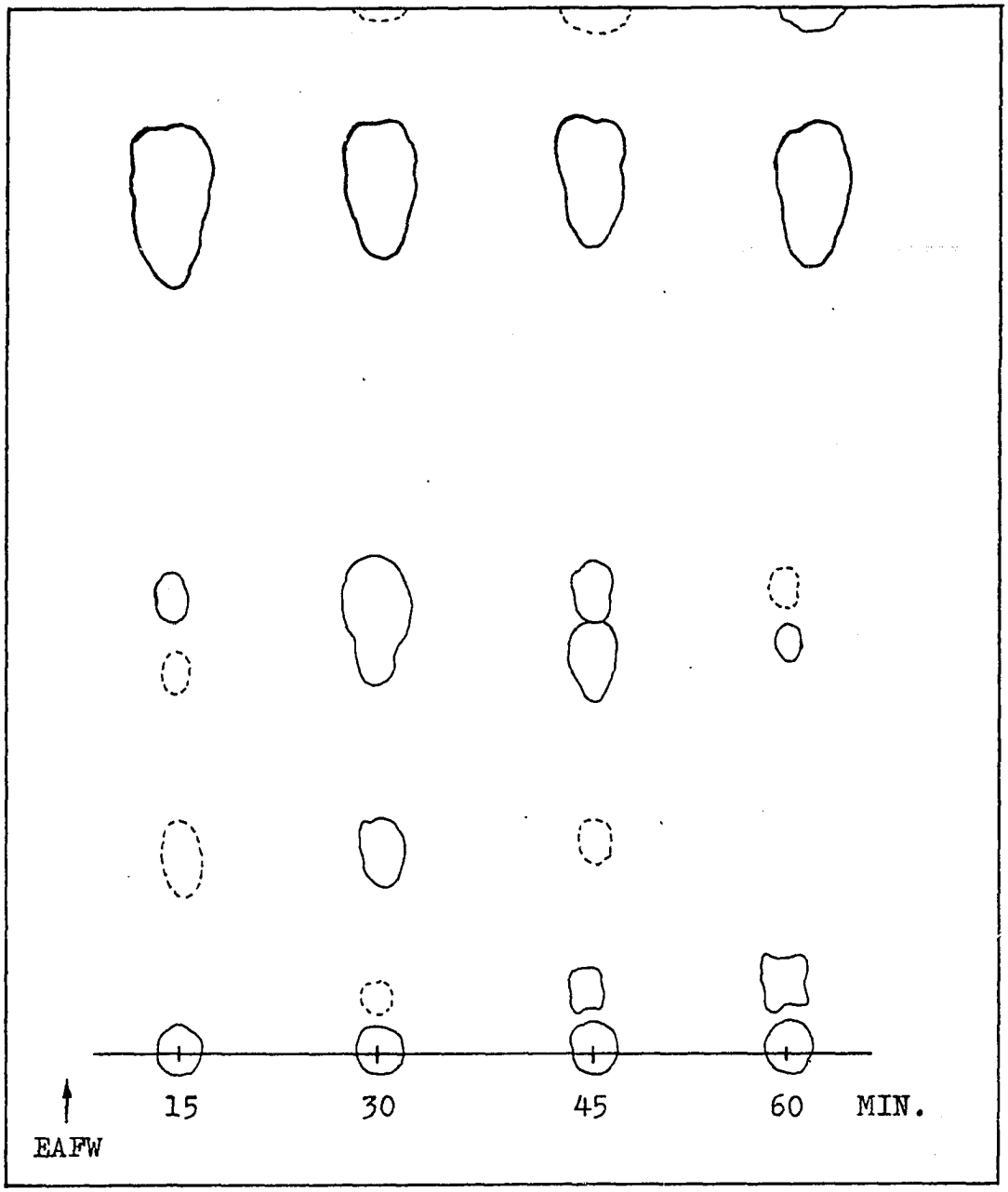


Fig. 3. Tracing of a radioautogram of DL-leucine-2-$C^{14}$ degradation

The radioautograms of the aerobic degradation of DL-leucine-2-$C^{14}$ confirmed the earlier results of the inactivity of the whole cells of B. leucinophagum on D-leucine.

When DL-leucine-2-$C^{14}$ was degraded in the absence of alkali to absorb evolved $CO_2$, a large number of radioactive spots was found on radioautograms. These were different than the low-activity compounds previously observed, however, and appeared to represent a variety of compounds formed as a result of reincorporation of leucine-derived $C^{14}O_2$. Since these obviously did not represent the primary degradation of leucine, no attempts to identify these compounds or explain their formation were made.

## $C^{14}$-L-leucine

To increase the specific activity of leucine degradation products as well as to insure detection of all compounds formed, since it was apparent that a cleavage of the molecule occurred, uniformly labelled $C^{14}$-leucine was used in all subsequent studies.

As before, washed whole cells were incubated aerobically at 30° C. in the presence of the substrate for varying periods of time, in this case 15 to 75 min. Fluted filter papers containing 0.1 ml. 20% KOH were placed in the center wells to absorb $CO_2$ to insure detection of compounds resulting from leucine degradation per se uncomplicated by side fixation reactions. To avoid destruction of any intermediate compounds

which might be unstable in the presence of acid, reactions were stopped by sudden freezing at -20° C. rather than by the use of trichloracetic acid. During analysis procedures the samples were thawed quickly in cold water and kept in an ice bath. For chromatographic analysis aliquots of the entire reaction mixture (including cells) were dried quickly on the paper under a gentle stream of warm air, care being taken that the temperature did not exceed 55° C.

Of all the chromatographic solvent systems tested, EAFW gave the best resolution of the greatest number of spots. None of the other solvents tested resolved radioactive compounds which did not correspond to one of the spots obtained with this solvent. EAFW possesses other advantages in addition: It is quickly and easily prepared; it does not separate into phases as do some other mixtures; it migrates very rapidly even when used in ascending technics; and it is very volatile and is quickly and completely removed from the paper after chromatographic development is completed. This solvent system was therefore adopted for routine exploratory use in all identification procedures.

The products of the aerobic degradation of $C^{14}$-L-leucine are shown in Fig. 4.

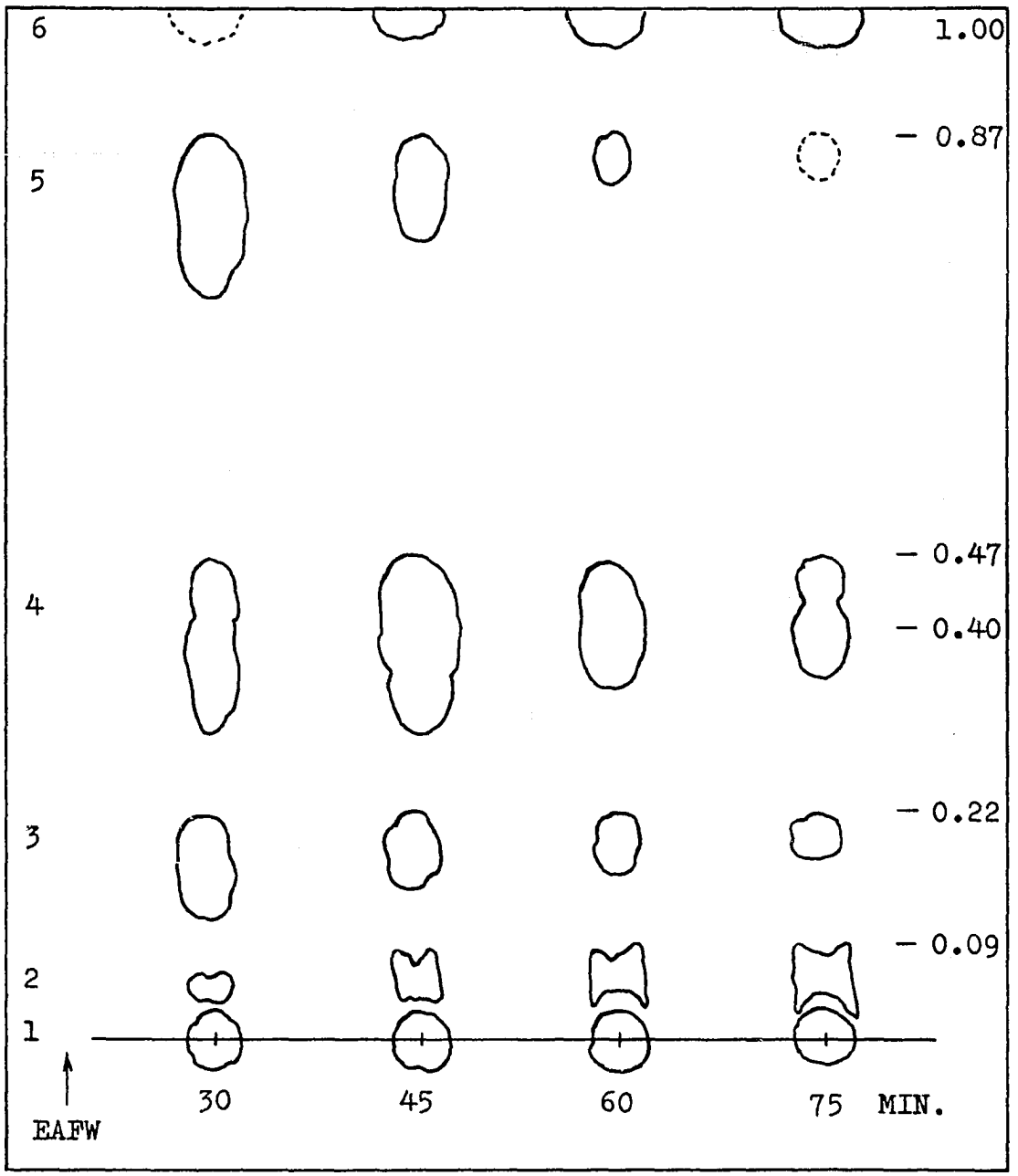


Fig. 4. Tracing of a radioautogram of $C^{14}$-L-leucine degradation

## Identification of Products

A large quantity of radioactive material remained at the origin (spot no. 1). This appeared to be largely protein or peptide since it gave characteristic ninhydrin and biuret color reactions. No radioactive compounds could be extracted from it with water, 80% ethanol or ether. To confirm its identity a quantity of the material isolated chromatographically was sealed into a small glass tube with 6 volumes of 6 N HCl and heated in the autoclave 4 hrs. at 121° C. The material was repeatedly evaporated to dryness to free it of residual HCl, dissolved in a small amount of 10% isopropanol, and subjected to chromatography and radioautography. A number of radioactive compounds -- all ninhydrin positive -- were detected and subsequently identified as amino acids (Table 9). No other compounds were present.

When whole cells, recovered from $C^{14}$-leucine degradation experiments, were washed rigorously and subjected to hydrolysis and identification, the same radioactive amino acids as listed in Table 9 were identified.

Preliminary analyses indicated that the majority of the compounds of Fig. 4 were ninhydrin-positive. Quantitative separations were carried out, the compounds located and recovered, and identified as described under "Materials and Methods."

Table 9. Identity of amino acids from spot no. 1

| Spot no. | Identity[a] |
|---|---|
| 1-a | Leucine |
| -b | Glutamic acid |
| -c | Aspartic acid |
| -d | Cysteic acid |
| -e | Serine |
| -f | Threonine |
| -g | Isoleucine |
| -h | Valine |

[a]Listed in order of apparent decreasing concentration.

Spot no. 2 was found to be mainly cysteic acid together with a small amount of cystine. No cysteine as such was found in degradation reactions when analyzed with EAFW or other acidic solvents. A small spot identified as cysteine was found, however, on chromatograms developed in EAW. Our experience has shown cysteine to be oxidized rapidly to cysteic acid during development in acidic solvents. A portion at least of the cysteic acid identified is thus due to spontaneous oxidation during analysis. This was confirmed by the observation that cysteine separated in EAW was isolated as cysteic acid when rechromatographed in EAFW. However, the

concentrations of cysteic acid found would appear to be too high to be accounted for completely on the basis of spontaneous oxidation. It would thus appear that cysteic acid was also being formed enzymatically during the experiment. This was later demonstrated (see below).

Spot no. 3 could be shown to be composed of a mixture of three compounds by development at lower temperatures (10° C.). Serine was found in highest concentration together with a small amount of threonine and a trace of a ninhydrin-negative compound which we have been unable to identify.

Spot no. 4 could similarly be shown to be composed of two compounds, glutamic and aspartic acids. On the basis of the area of the spot, which is proportional to concentration (18, p. 56), glutamate was generally found in higher concentrations than aspartate.

Spot no. 5 was composed solely of leucine.

We have been unable to identify the compound in spot no. 6. Quantitative recovery of this compound has been difficult, which suggests that it is perhaps very unstable or volatile. Although it is formed in relatively high concentrations, as judged by the intensity of spots on radioautograms, enough material could not be recovered to give satisfactory chemical classification tests.

In one series of experiments an additional ninhydrin-positive compound was found. This was conclusively

identified as taurine. Its detection in degradation experiments has not been consistent, however. The reason for this is not clear.

All of the products of $C^{14}$-leucine degradation are summarized in Table 10.

Table 10. Summary of products of $C^{14}$-leucine degradation

| Spot no. | $R_f$[a] | Identity |
|---|---|---|
| -- | -- | Carbon dioxide |
| 1 | 0 | Protein or peptide |
| | | Leucine<br>Glutamic acid<br>Aspartic acid<br>Cysteic acid (cysteine + cystine)<br>Serine<br>Threonine<br>Isoleucine<br>Valine |
| 2 | 0.99 | Cysteic acid + cystine + cysteine |
| 3 | 0.22 | Serine + threonine + unknown |
| 4 | 0.47 | Glutamic acid + aspartic acid |
| 6 | 1.00 | Unknown |
| (-- | 0.19 | Taurine) |

[a]In EAFW at 25° C.

## Time Series Experiments

The concentrations of the various products of leucine degradation varied with time during the course of the degradation. This could be deduced from both the areas and the densities of the spots on radioautograms. With the expectation that a knowledge of the sequence of the distribution of radioactivity would be of value in elucidating the pathway of degradation, quantitative analysis of the labelled products was attempted.

A number of methods are available for the quantitative estimation of compounds separated by paper chromatography (18, pp. 51-74). Three of these methods were evaluated, only one being successful in practice.

The concentration of a compound affects both the area and, in the case of light-absorbing substances, the optical density of the spots after development. Attempts were made to estimate concentrations by measurement of the areas of spots located on the radioautograms. This method was fraught with difficulties, however, in that very carefully standardized conditions during all phases of the analysis were required as well as the simultaneous inclusion of a number of internal controls on each chromatogram. For our purposes this technic was highly impractical. The second method, based on the optical densities of the darkened areas on

radioautograms, was somewhat more successful in that relative concentrations over a narrow range could be estimated. A number of factors interfered, however, such as inequalities in the transparency of the film due to scratches, finger smudges, incompleteness of clearing by hypo, water spots during drying, etc. Furthermore, above certain concentrations of isotope which blackened the film to the extent of complete opacity, the method could not be applied at all. It was impossible to remain within a range of useful optical densities and still detect radioautographically all the products of degradation.

The third method, although possessing some limitations and disadvantages, was of some value. After the radioautogram had been prepared, the radioactive areas were traced on the chromatogram, the spots excised, eluted and transferred to etched glass planchets using quantitative precautions. Two major difficulties were encountered. The protein or peptide material which remained at the origin was only partially recoverable, even when a variety of eluting solvents was used. Similarly, the unknown compound of spot no. 6 was not able to be recovered due either to its instability or volatility. For these reasons the data of Table 11, expressing the distribution of radioactivity from $C^{14}$-leucine degradation, must be interpreted with reservations.

With increased time of incubation spot no. 1 increased steadily in density. Since the reaction mixture constituted a growth medium, this was due apparently to $C^{14}$-leucine incorporation into protein or peptide as well as incorporation of other $C^{14}$-amino acids as they were formed. Leucine disappeared within 45 min. Spot no. 6 was barely discernible after 30 min., but then increased with continued incubation.

Table 11. Distribution of radioactivity from $C^{14}$-leucine degradation[a]

| Spot no. | Counts/min./spot[b] after 15 min. | 30 min. | 45 min. | 60 min. |
|---|---|---|---|---|
| 1[c] | 1280 | 1430 | 1860 | 2170 |
| 2 | 264 | 615 | 1490 | 1580 |
| 3 | 792 | 1760 | 528 | 182 |
| 4 | 1140 | 1950 | 707 | 530 |
| 5 | 4650 | 965 | 351 | 37 |
| 6[c] | 87 | 101 | 132 | 183 |

[a]The degradation mixture contained 100 µM tris, pH 7.0; 5 µM $C^{14}$-L-leucine (1.66 x $10^6$ dis./min., calcd. from spec. act.); 250 mg. wet cells in a total volume of 1.0 ml. Center well contained a fluted filter paper + 0.1 ml. 20% KOH. Incubated aerobically at 30.3° C. for the times shown. Reactions stopped by sudden freezing at -20° C.

[b]100 µl. originally applied at the origin; radioactivity eluted from each spot for counting after development in EAFW at 24° C.

[c]Data for these spots unreliable due to difficulties in recovery (see text).

Glutamate and aspartate were formed in highest concentrations early in the course of incubation (15 to 30 min.). accompanied by serine, then threonine. These then decreased, serine most rapidly, followed by glutamate and aspartate, with corresponding increases in concentrations of spot no. 6 and cysteic acid. The evolution of $C^{14}O_2$, estimated as described above, did not appear to increase after the first 30 min.

All of the above experiments on the degradation of leucine were carried out using washed whole cells. The reaction mixtures for these experiments actually constituted a growth medium since the organism was able to synthesize all of its protoplasmic constituents from leucine alone. Undoubtedly any scheme which attempts to explain the pathway of leucine metabolism on the basis of these results, therefore, will be complicated by a host of competing reactions which must be taken into consideration. In an effort to simplify these considerations and limit our investigations to the degradative reactions completely divorced from those synthetic reactions occurring in living cells, a number of enzyme preparations of B. leucinophagum were tested for their ability to metabolize $C^{14}$-L-leucine.

All attempts to prepare a cell-free extract active for the complete degradation of leucine have been unsuccessful, however. As described above, transamination experiments

utilizing cell-free sonic extracts of B. leucinophagum gave rise to a carbonyl compound which, when chromatographed in BEA as the 2,4-dinitrophenylhydrazone, gave rise to a single spot of $R_f$ 0.77. This was presumed to be the derivative of α-ketoisocaproic acid, the keto analog of leucine. When these experiments were repeated with $C^{14}$-leucine, the 0.77 spot was found to be highly radioactive. No other radioactive compounds could be detected, however. Sonic treatment for varying periods of time under a variety of conditions, both with and without centrifugation, rapid and slow drying of washed cells, grinding under a variety of conditions with powdered glass or alumina, treatment with lysozyme, solubilization with digitonin, and treatment with acetone at -20° C. -- all these methods have yielded either completely inactive extracts or extracts active on other substrates. No radioactive leucine degradation products other than α-ketoisocaproate could be detected under either aerobic or anaerobic conditions, however. These results would suggest that the intracellular organization and coupling of enzyme systems in this organism is very delicate, at least with respect to this particular substrate.

## Degradation of Leucine by Mammalian Reactions

Although little information is available in the literature concerning the complete degradation and utilization

of the carbon skeleton of leucine by microorganisms (40, p. 787), the pathways of metabolism in mammalian organisms have been studied thoroughly and are now well understood (57, p. 300). This sequence of reactions leads to the accumulation of acetoacetate, and is characterized by its dependence on the formation of intermediate compounds which are esters of Coenzyme A (CoA), and the fixation of $CO_2$ into $\beta$-hydroxyisovaleryl-CoA to form $\beta$-hydroxy-$\beta$-methylglutaryl-CoA. The latter compound is then cleaved specifically to yield acetoacetate and acetyl-CoA. It was logical, then, to determine whether evidence for this sequence of reactions could be obtained with B. leucinophagum.

Accumulation of acetoacetate

Acetoacetate is a compound which is rather unstable, and, like oxalacetate, decarboxylates spontaneously, especially in the presence of metallic ions and certain organic compounds. The decarboxylation product of acetoacetate is acetone. Both of these compounds possess a carbonyl group which is capable of reacting readily with 2,4-dinitrophenylhydrazine to form a characteristic derivative. Since 2,4-DNP derivatives are very easily detected, this method affords a sensitive means of detecting the presence of either acetoacetate or acetone.

Reaction mixtures from experiments on the degradation of leucine were deproteinized by the addition of trichlor-

acetic acid, and treated with a solution of 2,4-DNP in 0.5 N HCl. The sample was incubated for periods up to 24 hrs. at 37° C. to allow formation and precipitation of any 2,4-DNP derivatives, but negative results were consistently obtained. A sample of authentic acetoacetate treated under these conditions gave rise to heavy yields of yellow crystals which, when chromatographed in BEA, gave rise to a very pronounced spot at $R_f$ 0.63, with traces of another derivative at $R_f$ 0.98. When samples of authentic acetone were so treated, a single spot was located at $R_f$ 0.98 to 1.00.

When samples of the spent growth medium of B. leucinophagum were examined by the 2,4-DNP method, however, small amounts of the appropriate derivatives were detected (Fig. 5). These derivatives corresponded to acetoacetate and acetone. To determine whether they arose from leucine degradation, quantitative analyses were performed on growth medium supernatants over time periods ranging from 4 to 24 hrs. Although slight traces of the compounds were again detected, the concentration did not increase with time of incubation. Quantitative assays were carried out by the colorimetric measurement of the eluted 2,4-DNP's as well as by the colorimetric measurement of the colored diazo derivatives (93). Another experiment was performed in which the growth medium supernatants from cultures growing in the presence of $C^{14}$-leucine were analyzed for acetoacetate and/or acetone.

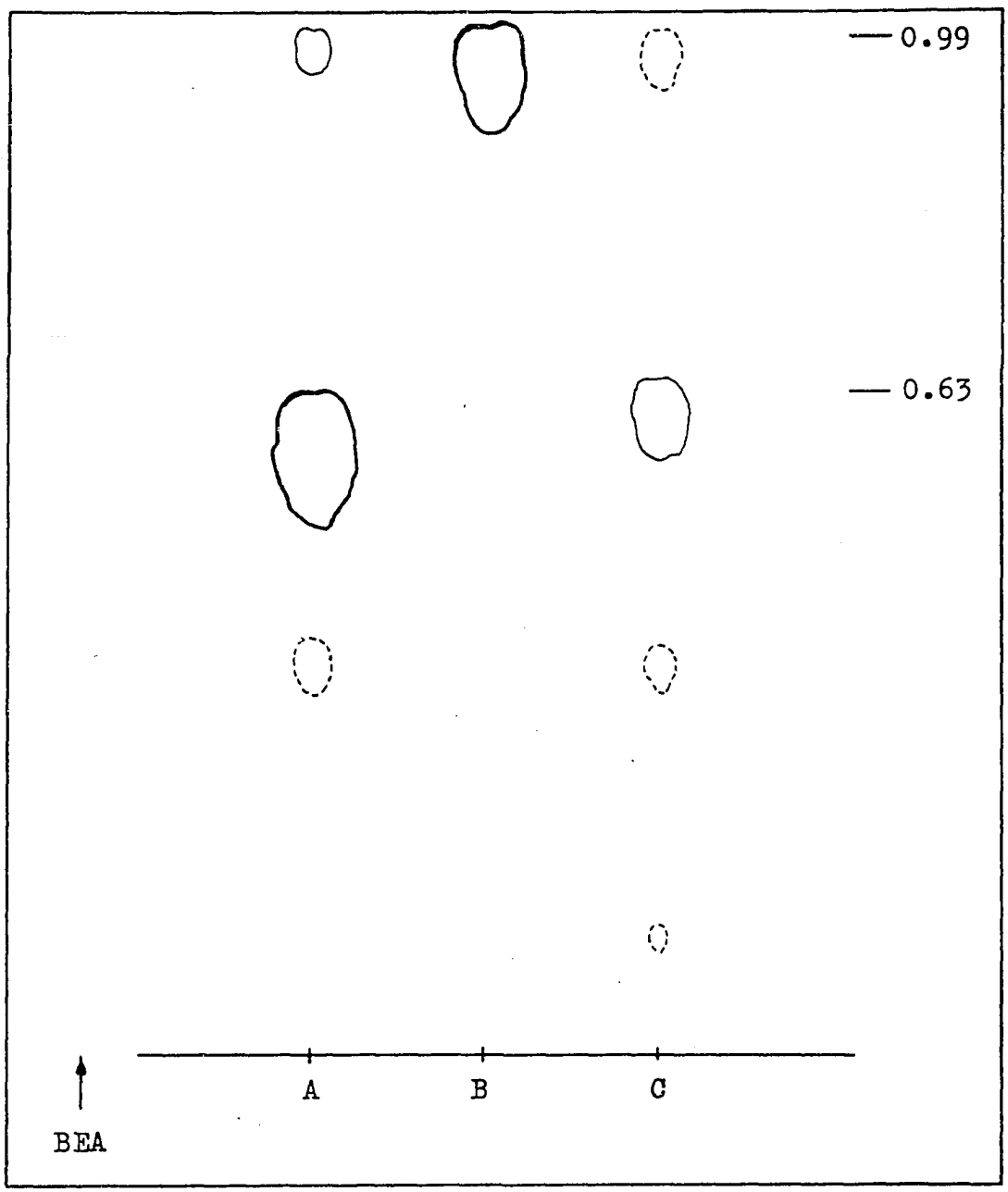


Fig. 5. Chromatographic detection of acetoacetate and acetone as the 2,4-DNP derivatives

A = authentic acetoacetate-2,4-DNP; B = acetone-2,4-DNP; C = supernate from leucine broth culture

Again, the appropriate derivatives were detected, but they were not radioactive. It must be concluded, therefore, that these traces of acetoacetate and acetone do not arise from the degradation of leucine, as in mammalian metabolism, but must be formed by other mechanisms.

Table 12 shows, furthermore, that washed whole cells are able to oxidize rapidly preparations of acetoacetate, but not acetone. In view of this, one would not expect acetoacetate to accumulate even if it were formed from leucine.

Table 12. Oxidation of acetoacetate and acetone[a]

| Time, min. | µl. Oxygen Uptake | |
|---|---|---|
| | Acetoacetate | Acetone |
| 10 | 35 | 0 |
| 20 | 71 | 1 |
| 30 | 107 | 3 |
| 40 | 143 | 5 |
| 50 | 183 | 8 |
| 60 | 206 | 8 |

[a]Each Warburg flask contained 100 µM tris, pH 7.0; 5 µM acetoacetate or acetone; 200 mg. wet cells in a total volume of 2.0 ml. Center well contained 0.1 ml. 20% KOH and a fluted filter paper. Temperature, 30.3° C.; gas phase, air. Data corrected for endogenous respiration (47 µl. $O_2$/hr.).

Coenzyme A ester formation

Reaction mixtures of leucine degradation experiments were examined for the presence of esters of CoA by the hydroxamic acid technic as well as by direct chromatography, followed by hydrolysis with alkali and spraying with the nitroprusside reagent of Toennies and Kolb, as described under "Methods." No evidence of CoA ester formation could be detected.

It was considered plausible that these chemical detection methods might not be sensitive enough to detect the minute quantities of CoA which might be present in unsupplemented reaction mixtures. In succeeding experiments, therefore, additional CoA was included during the degradation of leucine by washed whole cells, as well as by enzyme preparations as previously described. Again, no evidence of CoA ester formation was noted, and no free sulfhydryl compounds could be detected after alkaline hydrolysis, other than the CoA itself originally added to the mixture. Consistent, also, with previous results, was the fact that there was no evidence of leucine disappearance when an enzyme source other than washed whole cells was used.

Fixation of $CO_2$

The first step in the mammalian degradation of leucine is postulated to be the formation of $\alpha$-ketoisocaproate by

transamination (57, p. 300). The presence of a very active leucine- α-ketoglutarate transaminase was demonstrated with both whole cells as well as acetone-dried cells and cell-free sonic extracts, as previously described. α-Ketoglutarate was therefore routinely included in the following degradation experiments with cell-free juices in an attempt to stimulate the formation of α-ketoisocaproate and any subsequent reactions in which the latter compound might be involved.

A unique step in mammalian leucine degradation is the aerobic fixation of $CO_2$ into β-hydroxyisovaleryl-CoA (HIV-CoA) to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) (II). To determine whether this reaction occurred in the bacterial system under test, unlabelled L-leucine and α-ketoglutarate (as a possible source of eventual HIV-CoA) were incubated with $C^{14}O_2$ in the presence of either whole cells or cell-free extracts. Included in the reaction mixtures also were all of the necessary cofactors which had been demonstrated as essential for the same reaction in mammalian cell-free experiments. The results of a typical experiment are shown in Table 13.

These results show that a fixation of $CO_2$ did occur, but that it was inhibited under an atmosphere of air, in contrast to the reaction which occurs in pig heart and rat liver (10, 11). The fixation was also inhibited, but to a lesser extent, in the presence of phosphate. It has been

Table 13. Fixation of $CO_2$ in the presence of leucine[a]

| Expt. no. | Variable | cpm/ml.[b] | % of Control |
|---|---|---|---|
| 1 | Control | 730 | -- |
| 2 | Air atmosphere | 120 | 16 |
| 3 | Phosphate buffer; $N_2$ atmosphere | 440 | 60 |

[a]The control system (Expt. 1) contained 100 µM tris, pH 7.0; 25 µM L-leucine; 25 µM α-ketoglutarate; 200 µM $KHC^{14}O_3$ ($2 \times 10^7$ cpm/ml.); 25 µM cysteine; 2.5 µM ATP; 2.0 µM $CoA^3$; and crude cell-free extract (1.4 mg. protein) in a total volume of 1.0 ml. Reaction was run under 100% $N_2$ (except Expt. 2). In Expt. 3 100 µM phosphate, pH 7.0, were substituted for tris. The reaction was stopped by the addition of 0.2 ml. 50% trichloractic acid. Time = 60 min.

[b]100 µl. aliquots were dried on etched glass planchets and counted under an end-window GM tube.

observed on a number of occasions that a number of reactions of B. leucinophagum are slightly inhibited or take place at reduced rates in the presence of phosphate. The reasons for this are not clear; but to avoid the introduction of complicating factors tris buffers have therefore been used in the majority of experiments reported here. No such effects have been observed with tris buffers.

On the basis of these encouraging results, it was attempted to determine the factors upon which this observed fixation of $CO_2$ depended. The experiment represented by the data of Table 14 was designed to assess the effects of

elimination of one component of the reaction mixture at a time which had been determined as essential to the mammalian fixation reaction.

Table 14. Effect of various factors on $CO_2$ fixation[a]

| Omissions | cpm/ml. | % of Control |
|---|---|---|
| None | 2390 | -- |
| L-leucine | 1960 | 82 |
| α-ketoglutarate | 310 | 13 |
| L-leucine + α-ketoglutarate | 140 | 6 |
| Coenzyme A | 2080 | 87 |
| Adenosine triphosphate | 72 | 3 |
| Cysteine | 2240 | 94 |

[a]Conditions essentially the same as for Expt. 1, Table 13.

These results clearly show that 80 to 90% of the $CO_2$ fixation depends upon the presence of α-ketoglutarate, whereas only a small proportion is due to leucine (or derivatives thereof) in the absence of added α-ketoglutarate. The fixation was strongly dependent upon added ATP, as expected, but not CoA, however. The small amount of $CO_2$ fixed in the absence of both leucine and α-ketoglutatate was significant under the conditions of this experiment, and

was due to derivatives of leucine, apparently, because of the manner of preparation of the cells. (After growth in "Sta-mino" B, the cells were washed, then incubated in a 1% L-leucine solution for 2 hrs. at 30° C.). The products of the fixation reaction were clearly not acetoacetate. Acetoacetate is unstable and, like oxalacetate, decomposes readily and spontaneously. Upon recounting the planchets from the above experiment after storage for 24 hrs. at room temperature, essentially the same results were obtained. Furthermore, when the reaction mixtures were assayed chromatographically, no evidence of the formation of either HMG-CoA or its ultimate cleavage product, acetoacetate, was obtained. Thus, the reaction occurring here is not the $CO_2$ fixation reaction typical of mammalian leucine degradation.

The ability of these cell-free extracts of the test organism to fix $CO_2$ under these conditions was lost rapidly upon dialysis against distilled water (1 liter for 2 hrs. at 0° C.), and could not be restored by any of the cofactors listed in the table, nor by supplementation of reaction mixtures with diphosphopyridine nucleotide, lipoic acid, thiamine pyrophosphate, pyridoxal phosphate, $Mg^{++}$, $Zn^{++}$, or $Mn^{++}$.

Exchange reactions with $C^{14}O_2$

The fixation of $CO_2$ during mammalian leucine degradation into HIV-CoA to form HMG-CoA has been shown to be readily

reversible (10). In addition, Rudney, working with bakers' yeast (Saccarromyces cerevisiae) in a series of studies completely unrelated to leucine degradation, has shown that enzyme preparations were able to catalyze the condensation of acetate and acetoacetate (in the form of the CoA esters) to form HMG-CoA, which, in turn, is converted to HIV-CoA by decarboxylation (69).

It was considered that the negative results on $CO_2$ fixation previously described might possibly be explained by the fact that our methods of preparation of enzyme were too drastic and that, unwittingly, we had damaged the enzyme systems whose reactions we were attempting to demonstrate. In this case, evidence for a leucine-degrading pathway similar to that found in pig heart and rat liver, either positive or negative, might be obtained by attempting to demonstrate the fixation of $CO_2$ into an acetate-acetoacetate substrate system. If B. leucinophagum possessed enzymes capable of catalyzing the condensation of the CoA esters of acetate and acetoacetate to form HMG-CoA, which would then be decarboxylated reversibly, the presence of $C^{14}O_2$ in the reaction system would lead to the reversible incorporation of some of the isotope, which could then be detected, even at low levels, either in HMG-CoA or in its cleavage product, acetoacetate.

Accordingly, enzyme preparations by a variety of technics were incubated in the presence of acetate and/or acetoacetate,

CoA, $C^{14}O_2$ and the necessary cofactors shown to be essential for the mammalian and yeast reaction systems. Excess unreacted $C^{14}O_2$ was removed by the addition of acid at the end of the reaction period, and aliquots of the mixture were counted to determine incorporation of $C^{14}O_2$. Analysis by chromatography was carried out also to determine whether any $C^{14}$-products formed were HMG-CoA or acetoacetate. Consistent results were obtained with all enzyme preparations tested. The results of a typical experiment with sonic extracts are shown in Table 15.

Table 15. Fixation of $CO_2$ into the acetate-acetoacetate system[a]

| Omissions | cpm/ml. | % of Control |
|---|---|---|
| None | 3770 | -- |
| Acetoacetate | 3190 | 85 |
| Acetate | 1840 | 49 |
| Acetoacetate + acetate | 530 | 14 |
| CoA | 270 | 7 |
| ATP | 130 | 3 |

[a]The complete system contained 100 µM tris, pH 7.5; 0.5 µM $KHC^{14}O_3$ ($10^5$ cpm); 1.3 µM ATP; 1.0 µM CoA; 11.4 µM acetoacetate; 10 µM acetate; 25 µM cysteine; 0.5 ml. crude sonic extract (1.5 mg. protein) in a total volume of 1.30 ml. Reaction run at 30° C. 100 min. under 100% $N_2$. Stopped by addition of 0.2 ml. 50% trichloracetic acid.

These results show that the fixation of $CO_2$ in this system is dependent upon the presence of both ATP and CoA, in contrast to the fixation in the leucine system. The fixation is apparently dependent, furthermore, on the presence of acetate, rather that acetate plus acetoacetate. The fixation into acetoacetate can be explained by the presence in the sonic extract of acetoacetyl thiolase, the enzyme catalyzing the cleavage of acetoacetyl-CoA to acetyl-CoA, which is then involved in the $CO_2$ fixation. This hypothesis was supported by chromatographic evidence in that the same radioactive products were formed from both acetate and acetoacetate, except in lower concentration in the case of the latter substrate. The fixation products in this system were stable, as indicated by the fact that ageing the planchets at room temperature for 24 hrs. did not cause a reduction in the assay of radioactivity. This fact indicates that the product was not acetoacetate.

That the products of the fixation were not acetoacetate or HMG-CoA was confirmed by chromatographic examination of aliquots of the reaction mixtures. A number of radioactive products was detected, the same products whether acetate or acetoacetate was used as substrate, but these products were not carbonyl compounds (no radioactive 2,4-DNP derivatives were detectable). Therefore they were not acetoacetate, nor, when examined by the appropriate technics, did any of the

products correspond to HMG-CoA. No attempts at further identification of these products were made.

Dialysis of the cell-free extract resulted, as before, in rapid loss of $CO_2$-fixing activity in the acetate system and could not be restored by supplementation of reaction mixtures with any of the cofactors previously mentioned.

These results indicate that the $CO_2$ fixation reactions occurring here are not the same fixation reaction which occurs in mammalian tissue during the degradation of leucine, and, in fact, are different from each other in the acetate and the leucine systems.

Further evidence for the difference of the fixation reactions in the two systems is afforded by chromatographic examination of the products. These are not only neither HMG-CoA nor acetoacetate, but indeed differ from each other according to whether the leucine or the acetate system is examined.

The data of Table 16 provide further evidence for the differences between the two fixation systems. Although the data of Table 14 indicated that $\alpha$-ketoglutarate was the chief substrate of $CO_2$ fixation in the leucine system, a small but significant portion was due to leucine and/or its derivatives, though it is clear that this is not the reaction characteristic of the mammalian system. Table 16 shows that the leucine system is very sensitive to both pH and the

presence of atmospheric oxygen, whereas the acetate system is neither dependent on pH at the level tested nor inhibited by oxygen. To the contrary, the fixation of $CO_2$ is stimulated significantly aerobically in the acetate system.

Thus, the $CO_2$ fixation reactions occurring in the leucine and acetate systems differ from each other, and are not the same as the $CO_2$ fixation reaction which occurs in pig heart and rat liver during leucine degradation in these tissues.

Table 16. Comparison of the leucine and acetate $CO_2$ fixing systems

| Expt. no. | Variable[a] | cpm/ml. |
|---|---|---|
| 1 | Leucine system, pH 7.0 | 2780 |
| | pH 7.5 | 164 |
| 2 | Leucine system, anaerobic | 2780 |
| | aerobic | 334 |
| 3 | Acetate system, pH 7.0 | 4540 |
| | pH 7.5 | 4390 |
| 4 | Acetate system, anaerobic | 4540 |
| | aerobic | 5400 |

[a]Conditions as described previously except where indicated. Aerobic = air as gas phase; anaerobic = $N_2$ as gas phase.

In the absence of any evidence to the contrary, and on the basis of all the negative evidence cited above, it must

therefore be concluded that leucine is degraded by B. Leucinophagum under the conditions employed in our experiments by a sequence of reactions which differs from that found in the mammalian tissues studied by other workers.

It was necessary, then, to seek an explanation of the mechanism of formation of the products formed as the result of leucine degradation by whole cells of this organism.

During the characterization of B. leucinophagum it was observed that the organism was not only stimulated in its growth by the presence of free oxygen, but was in fact obligately aerobic (47). Also, glucose was metabolized to yield accumulations of free acid, and the organism grew readily and abundantly in the presence of citrate as sole carbon source and $NH_4^+$ as sole nitrogen source. Finally, the degradation of leucine-2-$C^{14}$ resulted in the production of $C^{14}O_2$, which indicates a cleavage of the leucine molecule, perhaps with the formation of a $C_2$ fragment which is then further metabolized to $CO_2$. All these facts suggest the presence and operation of a tricarboxylic acid (TCA) cycle.

The operation of a TCA cycle would thus account for the energy requirements of the organism during aerobic growth and a supply of intermediates for the synthetic reactions of cell growth from constituents of the medium such as citrate and $NH_4^+$. Furthermore, certain of the amino acids listed in Table 10, viz. aspartic acid and glutamic acid, are known to

be synthesized in microorganisms by a process of direct amination (or ammoniation) of their keto analogs, which are members of the TCA cycle. The formation of these compounds, as well as other members of the "aspartic-glutamic" families of amino acids (threonine and isoleucine, Table 10), would thus be explained (1).

## Evidence for a Tricarboxylic Acid Cycle

Moses has stated (60) that the only valid evidence for the operation of a TCA cycle is the measurement of flow rates and the recycling of compounds through the use of labelled substrates according to the methods of Roberts, et al. (65). Suggestive evidence for the presence and operation of the TCA cycle has been obtained by many other workers, however, by four general methods:

(a) growth in the presence of TCA cycle compounds and closely related compounds as the sole source of carbon;

(b) stimulation of oxidative activity by intermediates of the TCA cycle and closely related compounds;

(c) sensitivity to compounds known to inhibit reactions of the TCA cycle; and

(d) chemical conversion and accumulation of compounds known to be intermediates of the TCA cycle.

Accordingly, the following experiments were performed to obtain evidence for the presence and operation of the TCA cycle in B. leucinophagum.

Growth experiments

Growth media containing various members of the TCA cycle as sole carbon source were prepared as shown in Table 17.

Table 17. Growth media containing TCA cycle intermediates as sole carbon source

| Medium | Component | Concentration |
|---|---|---|
| Basal | Salts solution[a] | 1.0 ml./liter |
| | $NH_4H_2PO_4$ | 1.0 g./liter |
| A | Basal + acetate[b] | 3.0 g./liter |
| B | Basal + succinate | 3.0 |
| C | Basal + α-ketoglutarate | 3.0 |
| D | Basal + malate | 3.0 |

[a]Salts solution contained the following in mg./100 ml.: $K_2HPO_4.3H_2O$, 164 $KH_2PO_4$, 125; $MgSO_4.7H_2O$, 50; NaCl, 25; $FeSO_4,7H_2O$, 25; $MnSO_4$, 17. After standing 24 hrs. the slight precipitate was removed by filtration.

[b]TCA cycle compounds were dissolved in water, neutralized with KOH, filter-sterilized, and added to the sterile basal medium to give the final concentration shown.

The sterile media were dispensed in empty sterile test tubes which had been previously selected for their freedom from scratches and other imperfections which would interfere with transmittance measurements. The media were incubated 24 hrs. at 30° C. before inoculation to insure their sterility. They were then inoculated with one drop of a suspension of washed cells grown 24 hrs. at 30° C. in a medium containing salts and 1% L-leucine as sole carbon and nitrogen source. After an initial reading at 0 time, the tubes were incubated at 30° C. and read in the spectrophotometer at intervals of 4 hrs. Three replicates were included in each experiment. The results are shown in Fig. 6.

All tubes showed rapid and abundant growth with minimal lag periods, as would be expected with cultures already possessing a complement of enzymes for complete utilization of the growth substrates. Acetate and α-ketoglutarate were utilized initially at much greater rates than succinate and malate, although after longer incubation periods there was approximately the same amount of total growth in all tubes.

A similar experiment was designed to test leucine-grown cells for their ability to utilize amino acids (alanine, aspartate and glutamate), closely related chemically to members of the TCA cycle, as sole source of both carbon and nitrogen. These results are shown in Fig. 7.

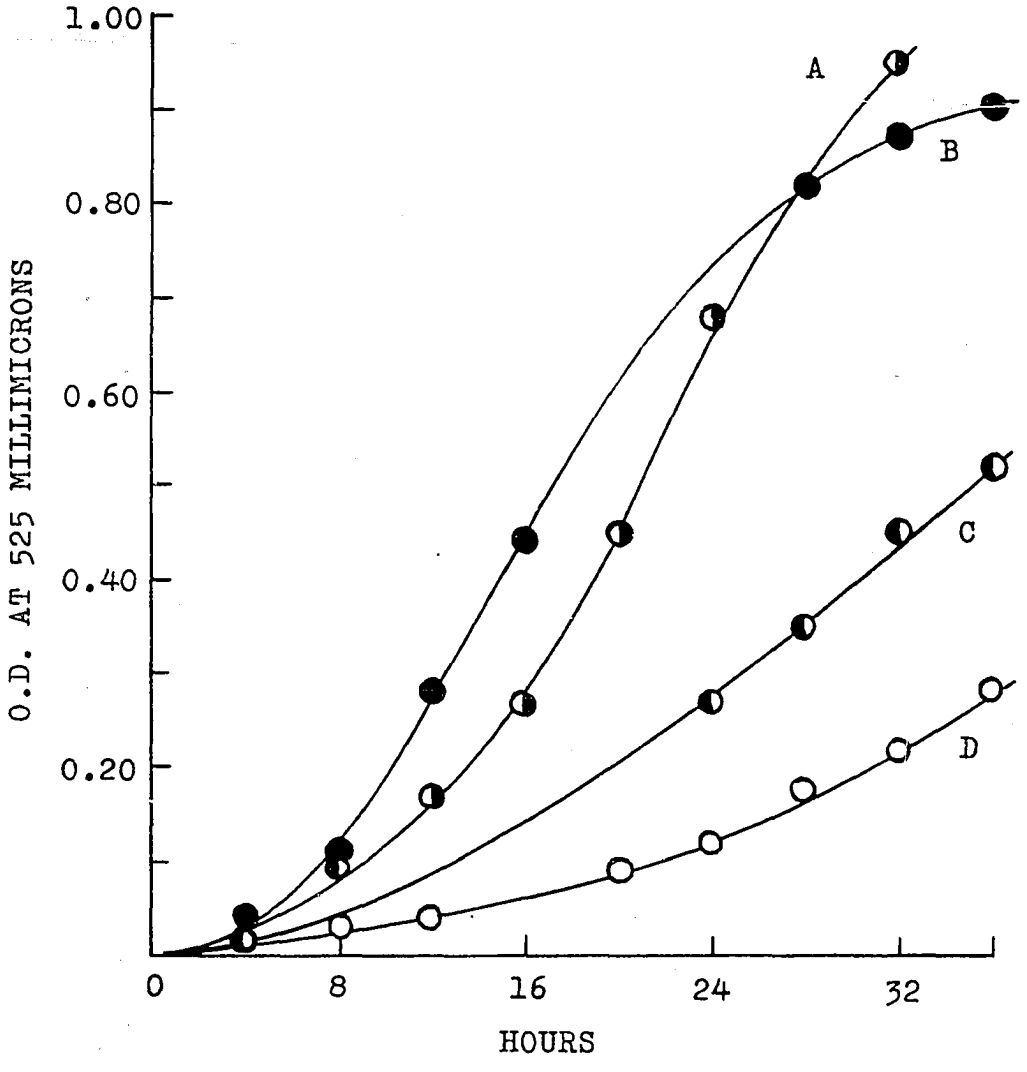


Fig. 6. Growth on TCA cycle compounds as sole carbon source

A = acetate; B = $\alpha$-ketoglutarate; C = succinate
D = malate

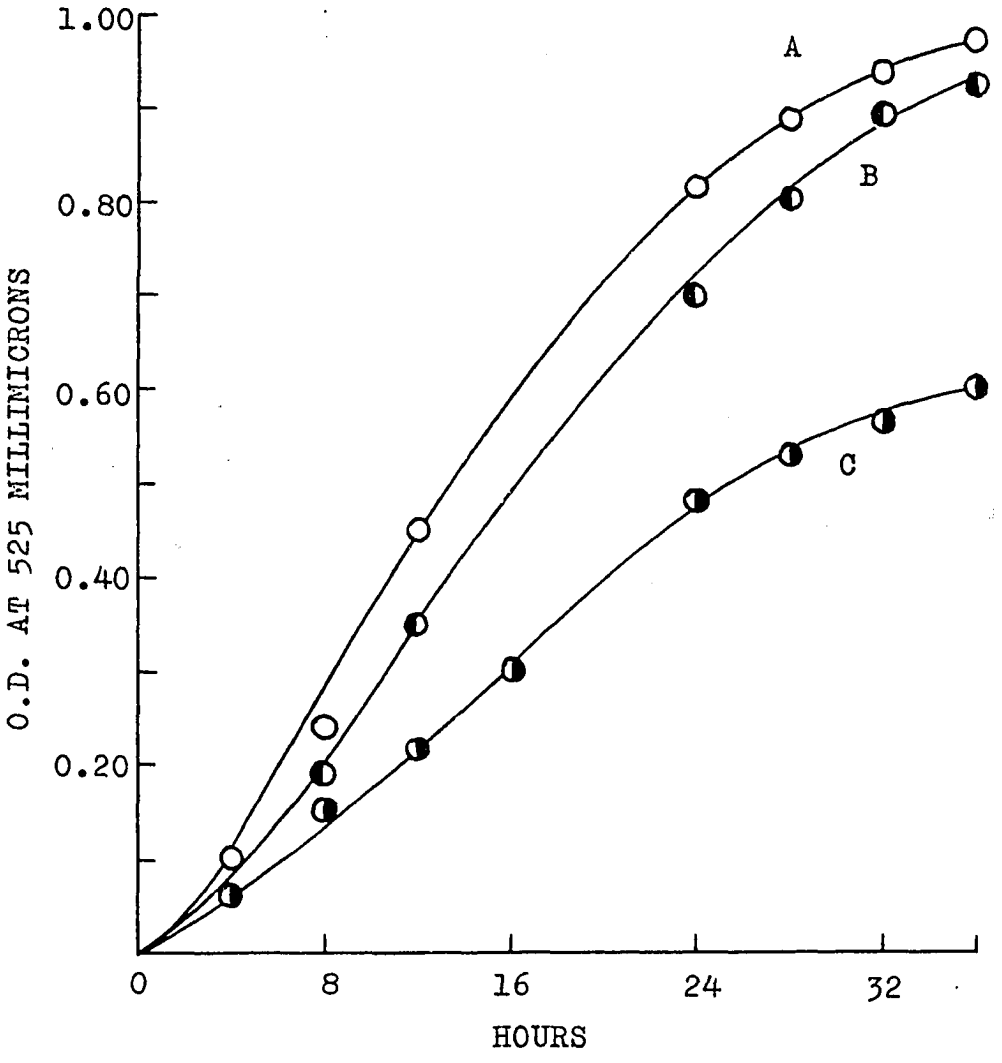


Fig. 7. Growth on single amino acids as sole carbon and nitrogen source

A = aspartate; B = glutamate; C = alanine

Again, rapid growth resulted, indicating that no prolonged lag period was required for adaptive synthesis of enzymes capable of utilizing the growth substrates for synthetic reactions.

Oxidation of TCA cycle compounds

Manometric experiments were next conducted to determine the ability of members of the TCA cycle to stimulate the oxidative activity of washed whole cells of the test organism. The direct method was employed in which a fluted filter paper containing 0.1 ml. 20% KOH was placed in the center well of the Warburg flasks to absorb envolved $CO_2$. The results of a typical experiment measuring oxygen uptake on $\alpha$-ketoglutarate, malate, citrate, fumarate, succinate and acetate are shown in Table 18.

It can be seen that all these compounds readily stimulated the uptake of oxygen by washed whole cells and immediately with no initial lag periods, indicating that the cells have no permeability barriers to entrance of these compounds into the cells, and that an active complement of enzymes is present for the oxidative metabolism of these TCA cycle intermediates.

Effects of TCA cycle inhibitors

One of the classical metabolic inhibitors is malonic acid, a compound which has long been known to inhibit the

Table 18. Oxygen uptake on TCA cycle compounds[a,b]

| Time, min. | µl. Oxygen Uptake on AKG | MAL | CIT | FUM | SUC | ACE |
|---|---|---|---|---|---|---|
| 10 | 52 | 32 | 24 | 28 | 8 | 28 |
| 20 | 97 | 78 | 54 | 66 | 40 | 81 |
| 30 | 118 | 131 | 95 | 116 | 74 | 130 |
| 40 | 133 | 182 | 132 | 166 | 102 | 186 |
| 50 | 144 | 228 | 166 | 208 | 152 | 220 |
| 60 | 155 | 264 | 199 | 242 | 174 | 243 |
| 70 | 164 | 291 | 237 | 264 | 178 | 249 |
| 80 | 171 | 304 | 266 | 274 | 192 | 265 |

[a]Warburg flasks contained 100 µM tris, pH 7.0; 5 µM of the substrate indicated; and 150 mg. wet cells in a total volume of 2.0 ml. Center well contained a filter paper and 0.1 ml. 20% KOH. Data corrected for endogenous respiration.

[b]AKG = α-ketoglutarate; MAL = malate; CIT = citrate; FUM = fumarate; SUC = succinate; ACE = acetate. The compounds were employed as the potassium salts.

TCA cycle at the point at which succinic acid is oxidized to fumaric acid (40, p. 502). The action of malonate on succinic dehydrogenase is known to be competitive.

In seeking evidence for a TCA cycle in B. leucinophagum it was logical, therefore, to determine the effect of malonate on succinic acid oxidation. Accordingly, washed whole cells were incubated in the presence of succinic acid

and equimolar concentrations of malonic acid, both compounds being employed in the form of their potassium salts. The results of such an experiment are shown in Table 19.

Table 19. Effect of malonate on succinate oxidation by whole cells[a]

| Time, min. | µl. Oxygen Uptake on Succinate with malonate | without malonate |
|---|---|---|
| 10 | 103 | 113 |
| 20 | 231 | 258 |
| 30 | 287 | 316 |
| 40 | 318 | 344 |
| 50 | 364 | 373 |
| 60 | 413 | 398 |
| 70 | 475 | 422 |
| 80 | 520 | 437 |

[a]Each flask contained 100 µM tris, pH 7.0; 12 µM succinate and 12 µM malonate where indicated; 200 mg. wet cells in a total volume of 2.0 ml. Center well contained a filter paper and KOH. Data corrected for endogenous respiration. Temperature = 30.3° C.; gas phase = air.

These data show that whole cells oxidized succinate very rapidly, as before, but contrary to expectations, malonate appeared to have absolutely no effect on the rates of succinate oxidation. Indeed, after lag periods of some

40 min. malonate appeared to be oxidized, slowly at first, but then at increasing rates.

Moses (59) and other workers have suggested that this ineffectiveness of malonate for succinic dehydrogenase inhibition with whole cells might be due to permeability effects since it can sometimes be overcome by altering the pH of the test system or by the use of esters of malonic acid (13).

To determine whether this explanation was valid in the present instance, the experiment represented by the data of Table 19 was repeated over a wide range of pH values. Tris buffers were used over the range pH 7.0 to 9.0 at increments of 0.5 pH. Phosphate buffers were used over the range pH 6.0 to 8.0 at increments of 0.4 pH. Phthalate buffers were employed at pH 4.0, 4.5 and 5.0. No essential differences were noted with whole cells in the tris and phosphate buffers at any of the pH values tested. Whole cells were completely inactive in phthalate buffers between pH 4 to 5.

When the above experiment was repeated, however, with acetone-dried cells rather than washed whole cells, malonate strongly inhibited oxygen uptake on succinate as well as on a number of other intermediates of the TCA cycle tested. This is shown in Table 20. Furthermore, no oxidative activity of acetone-dried cells on malonate could be detected as in whole cells.

Table 20. Effect of malonate on TCA cycle compounds oxidation by acetone-dried cells[a]

| Substrate | µl. Oxygen/hr. (-) malonate | (+) malonate | Per cent Inhibition |
|---|---|---|---|
| Acetate | 378 | 108 | 71.4 |
| Succinate | 200 | 11 | 94.5 |
| Fumarate | 259 | 37 | 85.7 |
| Malate | 232 | 5 | 97.8 |
| Malonate | --- | 4 | --- |

[a]Each flask contained 100 µM tris, pH 7.0; 10 µM substrate; 0.3 ml. acetone powder suspension (equivalent to 500 mg. wet cells) in a total volume of 2.0 ml. Center well contained a fluted filter paper and alkali. Shaken under air at 30.3° C. Data corrected for endogenous respiration (35-49 µl./hr.).

In view of the unexpected results obtained with malonate and whole cells of B. leucinophagum, the competitive nature of the malonate inhibition with acetone-dried cells was investigated. A truly competitive inhibition is easily reversed by increasing concentrations of the substrate in the presence of the inhibitor. This is shown in the case of the fumarate-malonate system with acetone-dried cells (Table 21).

The ability of whole cells of B. leucinophagum to oxidize malonate possesses a number of interesting features. First, malonic acid is ordinarily considered to be a

Table 21. Reversal of malonate inhibition of fumarate oxidation[a]

| Fumarate, μM | Malonate, μM | μl. Oxygen/hr. | Per cent Inhibition |
|---|---|---|---|
| 2.0 | -- | 257 | (Control) |
| 2.0 | 2.0 | 49 | 81.0 |
| 10.0 | 2.0 | 115 | 55.3 |
| 20.0 | 2.0 | 192 | 25.3 |
| 30.0 | 2.0 | 219 | 14.8 |
| 40.0 | 2.0 | 227 | 11.7 |

[a]Conditions the same as for Table 20, except that 0.4 ml. acetone powder suspension (equivalent to 700 mg. wet cells) used.

metabolic inhibitor, and few species are able to utilize it readily. To the writer's knowledge the only other organisms which are able to metabolize malonic acid are members of the genus Pseudomonas, Gram negative organisms found widely distributed in Nature in the soil, among other habitats. Furthermore, the ability of B. leucinophagum to oxidize this unusual substrate at increasing rates after lag periods of 40 to 50 min. suggests strongly an adaptive process. Previous chromatographic analyses of leucine degradation by whole cells indicated that B. leucinophagum, like other Gram positive microorganisms, possesses a significant intra-

cellular pool of free amino acids which can be readily mobilized for adaptive protein synthesis. Since the inhibitor experiments described above with whole versus acetone-dried cells support the hypothesis of a permeability barrier to malonate, three possibilities for adaptive synthesis present themselves: (a) that a "malonate-oxidizing" system is formed adaptively in response to induction; (b) that the malonate-oxidizing system is already present and intact in the cells, but the substrate is unable to reach it until a "malonate permease" is formed adaptively (24); and (c) a combination of both (a) and (b). A thorough investigation of this interesting problem is beyond the scope of the present work, but the data of Table 22 support the hypothesis of an adaptive response to malonate.

A quantity of cells was grown in the usual growth medium supplemented with 0.1% malonate. These malonate-grown cells were then compared with normal (unadapted?) cells for their ability to oxidize malonate. The effect of chloramphenicol at a concentration of 200 µg./ml., a potent inhibitor of de novo protein synthesis (97), was also assessed by the usual manometric technics.

These data show that not only is malonate oxidized at much higher rates by malonate-grown cells, but the oxidation by these cells is much less sensitive to the action of

Table 22. Malonate oxidation by normal and malonate-grown cells and the effect of chloramphenicol[a]

| Variable | µl. Oxygen/hr. (-) CMP[b] | (+) CMP | Per cent Inhibition |
|---|---|---|---|
| Normal cells | 67 | 21 | 68.6 |
| Malonate-grown cells | 198 | 156 | 21.2 |

[a]Each flask contained 100 µM tris, pH 7.0; 10 µM malonate; and chloramphenicol (200 µg./ml.) where indicated. Total volume 2.0 ml.; gas phase = air; temperature = 30.3° C. Data corrected for endogenous respiration: normal cells = 54 µl. $O_2$/hr; malonate-grown cells = 39 µl. $O_2$/hr.

[b]CMP = chloramphenicol.

chloramphenicol. These data would thus lend support the hypothesis of an adaptive enzyme synthesis.

Arsenite is another potent inhibitor of the TCA cycle. This compound, although less specific in its action, is known to inhibit the oxidative decarboxylation of α-keto-glutarate to succinyl-CoA. Table 23 shows that oxygen uptake by acetone powders in the presence of acetate, succinate and malate was inhibited.

Another inhibitor of the TCA cycle is fluoroacetate. This compound replaces acetate in the condensation with oxalacetate, to form fluorocitrate, which cannot be metabolized by aconitase (53). When fluoroacetate was added to a suspension of acetone-dried cells oxidizing acetate, a rapid

Table 23. Inhibition by arsenite of TCA cycle oxidations[a]

| Substrate | µl. Oxygen/hr. (-) arsenite | (+) arsenite | Per cent Inhibition |
|---|---|---|---|
| Acetate | 471 | 108 | 77.1 |
| Succinate | 319 | 42 | 86.8 |
| Malate | 267 | 43 | 83.9 |

[a]Each flask contained 100 µM tris, pH 7.0; 10 µM substrate; 10 µM arsenite where indicated; and 0.3 ml. acetone powder suspension (equivalent to 500 mg. wet cells) in a total volume of 2.0 ml. Center well contained alkali. Shaken under air at 30.3° C. Data corrected for endogenous respiration (38 µl. $O_2$/hr.).

decrease in the rate of oxidation was noted (Fig. 8). Fluoroacetate itself was not significantly oxidized by acetone-dried cells of B. leucinophagum.

Conversion of TCA cycle compounds

Further evidence for the occurrence of reactions characteristic of the TCA cycle is afforded by chromatographic analyses of reaction mixtures containing the above TCA cycle inhibitors.

Large amounts of succinic acid were found to accumulate in the presence of malonate and malate, fumarate or acetate. When arsenite was included in mixtures containing malate, succinate or acetate, a carbonyl compound accumulated which,

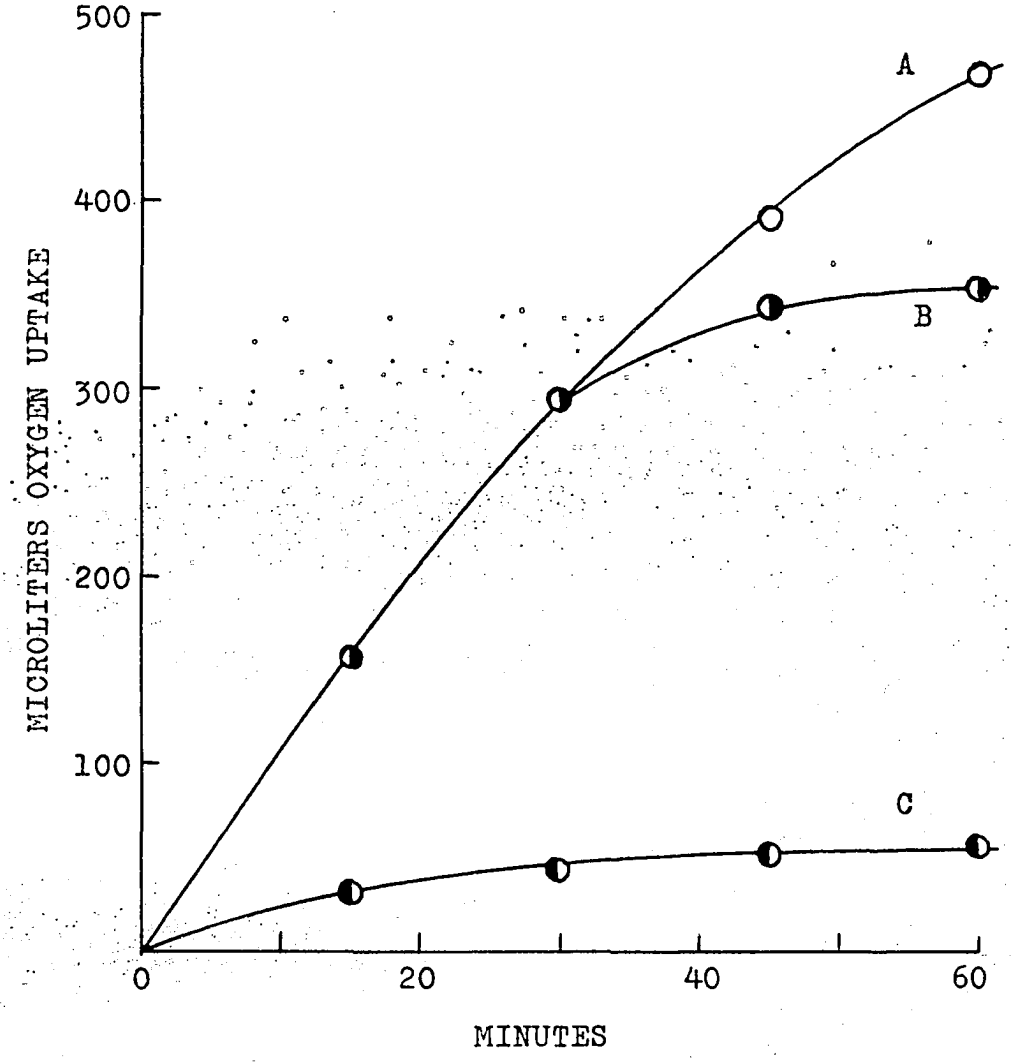


Fig. 8. Inhibition of acetate oxidation by fluoroacetate

A = acetate alone; B = acetate + fluoroacetate at 30 min.;
C = fluoroacetate alone

when separated as the 2,4-DNP derivative, possessed the same $R_f$ as the derivative of authentic α-ketoglutarate.

The observations reported here furnish strong supporting evidence for the hypothesis that a TCA cycle is present and operative when B. leucinophagum is growing in the presence of L-leucine. Thus the formation of the amino acids of the aspartic-glutamic families (aspartic and glutamic acids, threonine and isoleucine), listed in Table 10, can be explained by mechanisms in conformity with reactions known to occur in other organisms (1, 57).

## Reactions Involving Cysteine

Cysteine, the probable precursor of cystine and cysteic acid in biosynthetic reactions (57, p. 321), is known to be synthesized in microorganisms in two ways: by a reversal of the cysteine desulfhydrase reaction, and by the socalled cystathionine pathway (57, p. 307).

It had often been observed that suspensions of whole cells which had been allowed to age for long periods of time in the refrigerator often assumed an odor which bore unmistakable characteristics of the odor of hydrogen sulfide. When strips of filter paper, impregnated with ferrous sulfate or lead acetate were suspended above these ageing cell suspensions, they were gradually darkened, suggesting by this simple qualitative test that $H_2S$ was indeed being given off.

In an attempt to determine whether the cysteine desulfhydrase occurs in B. leucinophagum, washed whole cells were incubated in the presence of cysteine for varying periods and the reaction mixtures analyzed as previously described for cysteine disappearance. The results of such an experiment are shown in Table 24.

Table 24. Disappearance of cysteine[a]

| Time, min. | Cysteine remaining µM/ml. | Cysteine utilized[b] µM/ml. |
|---|---|---|
| 0 | 24.3 | --- |
| 15 | 19.7 | 5.3 |
| 30 | 14.1 | 10.9 |
| 45 | 12.7 | 12.3 |
| 60 | 10.9 | 14.1 |

[a]Reaction mixtures contained 100 µM tris, pH 7.0; 25 µM cysteine; and 200 mg. wet cells in a total volume of 1.0 ml. Reaction run at 30.3° C. under air, and stopped by the addition of 0.2 ml. 50% trichloracetic acid.

[b]By difference.

Cysteine was apparently utilized at significant rates, but inclusion of filter papers in the center wells of the reaction vessels moistened with ferrous sulfate, lead acetate, or even the more sensitive silver nitrate, showed no evidence of darkening. Chromatographic analysis did show, however, that considerable quantities of cysteic acid were

detectable after development in EAW. Traces of cystine were also noted (Fig. 9). When the experiment was repeated under anaerobic conditions under an atmosphere provided by flushing the vessels with 100% $N_2$, filter papers placed in the center well containing 0.1 ml. 1% $AgNO_3$ turned brownish-black. No evidence of pyruvate, the other product of this reaction, could be detected, however, even when reaction mixtures were treated suddenly with solutions of 0.5% 2,4-DNP in 6 N HCl to halt the reactions. It must be concluded, therefore, that pyruvate is far too reactive an intermediate to accumulate under these experimental conditions.

When the above experiment was repeated with suspensions of acetone-dried cells, similar results were consistently obtained, except that in this case slightly larger spots of cystine were noted on paper chromatograms of reaction mixtures developed in EAW.

Table 25 shows the results of a manometric experiment comparing the rates of oxidation of cysteine by whole cells and acetone-dried cells. Approximately two-thirds of the activity appears to be lost by acetone treatment of the cells with respect to this substrate.

These results suggest that cysteine is the precursor of both the cystine and the cysteic acid identified during the degradation of leucine by B. leucinophagum (Table 10).

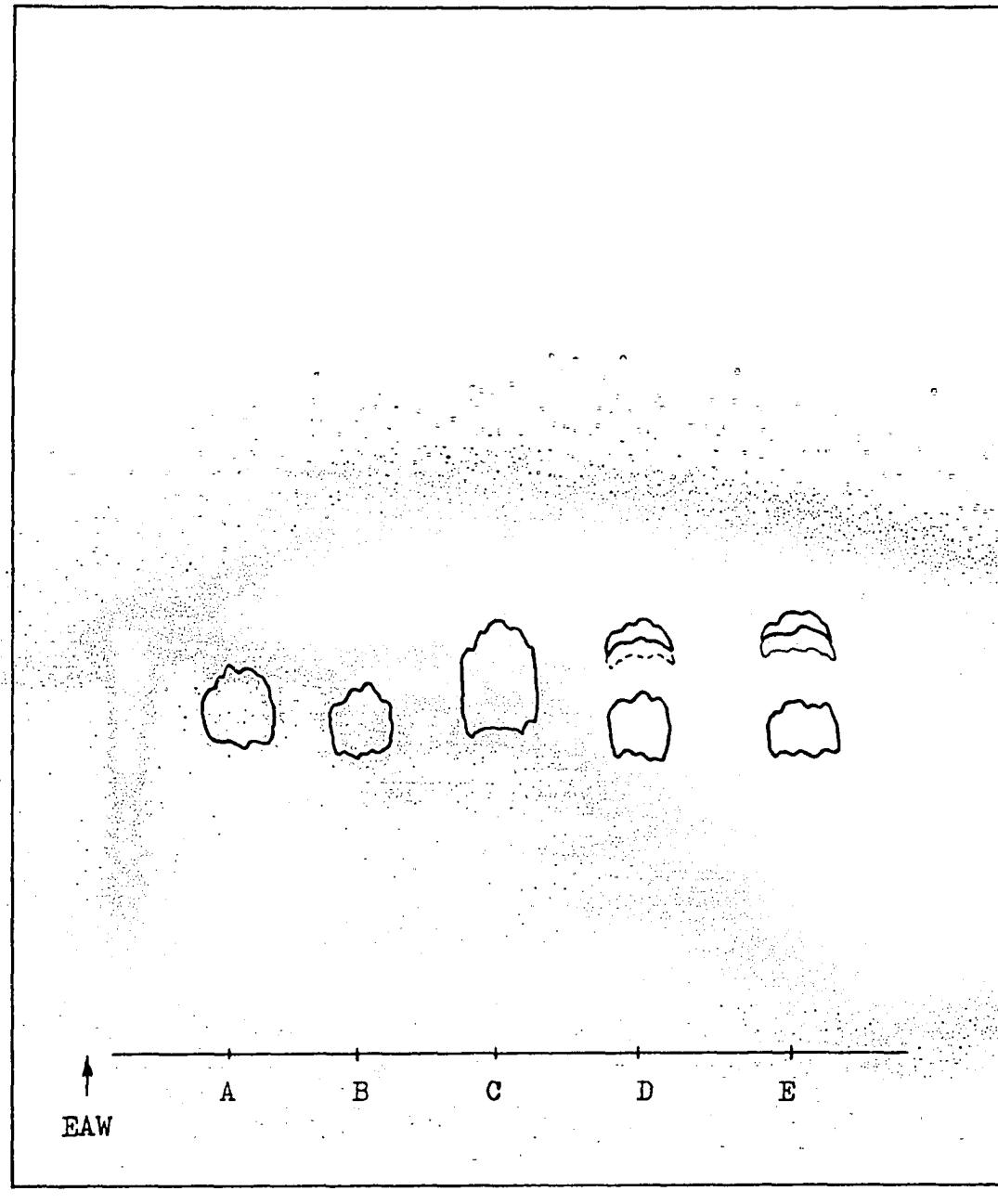


Fig. 9. Chromatographic evidence of formation of cystine and cysteic acid from cysteine

A = cysteine; B = cysteic acid; C = cystine; D = whole cell reaction mixture; E = acetone powder reaction mixture

The formation of these compounds thus appears to conform to the mechanisms previously observed to occur in other micro-organisms.

Table 25. Oxidation of cysteine[a]

| Time, min. | µl. Oxygen Uptake by Whole cells | Acetone-dried cells |
|---|---|---|
| 10 | 52 | 17 |
| 20 | 112 | 36 |
| 30 | 172 | 55 |
| 42 | 222 | 73 |
| 50 | 258 | 80 |
| 60 | 284 | 98 |

[a]Each flask contained 100 µM tris, pH 7.0; 10 µM cysteine; 150 mg. wet cells or the equivalent as an acetone powder suspension in a total volume of 2.0 ml. Center well contained a fluted filter paper + 0.1 ml. 20% KOH. Shaken under air at 30.3° C. Data corrected for endogenous respiration (whole cells = 62 µl. $O_2$/hr.; acetone-dried cells = 9 µl. $O_2$/hr.).

## DISCUSSION

The genus Brevibacterium Breed was established in 1953 (20), and together with the genus Kurthia Trevisan constitutes the family Brevibacteriaceae Breed (21). The properties of this species, described above, indicate that it is properly classified in the family Brevibacteriaceae Breed according to the 7th edition of Bergey's Manual. The two genera which comprise this family, Brevibacterium and Kurthia, are differentiated primarily by the fact that members of the latter genus form long, unbranched rods or filaments and do not utilize carbohydrates. The members of Brevibacterium are typically short, unbranched, Gram positive, non-sporulating, rod-shaped bacteria. The type species is B. linens (Weigmann) Breed.

Although long, unbranched rods have been observed in cultures of the organism described here, these occurred only at temperatures above the optimum, and have never been observed at lower temperatures. In addition, these filamentous forms are accompanied by the presence of distorted, swollen and club-shaped cells which are typical of the pleomorphic forms found in bacterial cultures exposed to degenerating influences. Serial microscopic examination of developing cultures showed no evidence that these filaments were formed first and then divided into coccoid elements, as is described for the genus Kurthia.

Although 23 species of Brevibacterium are listed in the 7th edition of Bergey's Manual, a careful comparison of cultural, morphological and physiological characteristerics showed that the organism described here is not identical to any of these. This species is nonmotile; therefore it is not B. incertum, B. imperiale, B. lipolyticum, B. acetylicum, B. sulfureum or B. helvolum. It produces no pigments when grown on agar, and is therefore not B. linens, B. erythrogenes, B. fulvum, B. insectiphilium, B. brunneum, B. vitarumen, B. maris or B. fuscum. Other properties differ sufficiently also to show that it is not a pigmentless variety of these species. Nitrites are not produced from nitrates; therefore this species is not B. stationis, B. quale or B. ammoniagenes. B. leucinophagum does not liquefy gelatin, as does B. sociovivum, B. immotum, and B. marinopiscosum. Furthermore, sea water or similar saline solutions are not required to initiate growth. The organism described here is not identical to B. minutiferula since the latter produces slight acidity from sucrose and in litmus milk. B. leucinophagum possesses none of the cultural characteristics of B. healii. Finally, it produces acid from glucose only, whereas B. tegumenticola produces acid from glucose, maltose and sucrose.

On the basis of all this evidence, therefore, the proposal of a new species was warranted.

The organism appeared to be well suited to the requirements of the present investigation since it grew rapidly and abundantly on a variety of simple media, it remained viable for long periods with infrequent transfer, and it obviously degraded the leucine molecule completely since L-leucine served adequately as sole carbon and nitrogen source. Later experience showed, however, that the organism must possess a very delicate intracellular organization which was particularly susceptible to damage since all attempts to prepare enzymes for complete leucine degradation in a cell-free state were unsuccessful. The obligatory use of whole cells, therefore, imposed a very serious disadvantage which was further aggravated by the fact that in the presence of leucine the cells were not able to be maintained in a resting state. This use of growing cells, with all their concomittant synthetic reactions, must be kept in mind during the interpretation of the experimental results obtained. It is quite possible that the nature of the metabolic pathway might thus be obscured or even altered from that found in non-growing cells. For example, the inability to detect the products of typical decarboxylation and deamination reactions in our reaction mixtures does not necessarily mean that these reactions do not occur; it means simply that these products did not accumulate under our experimental conditions.

The reasons for the slow rates of leucine utilization represented by the data of Table 1 are not clear. It would appear that such slow rates of utilization of substrate serving as sole source of carbon and nitrogen would not be compatible with normal growth rates. It can only be concluded that treatment of the cells for the experiment described must have altered the cells in some subtle way, perhaps by leaching essential intermediates during washing procedures, so as to affect the normal rates of substrate assimilation. The later observations of the dependence of leucine degradation on the presence of aerobic conditions will partially explain this phenomenon also in that the deep tubes used in the experiment did not permit adequate aeration of the test system.

In conformity with observations on a number of other biochemical systems, B. leucinophagum is not able to utilize the D-isomer of leucine. The L-isomers of amino acids are those which are commonly found to be incorporated into native protein, although the investigations of other workers in recent years have demonstrated the importance of the role of the D-isomers also in normal metabolism. However, not only are the D-isomers of amino acids less commonly utilized, but they often act as inhibitors of L-isomer metabolism, probably because of steric hindrance. This inhibition is evident in the present instance also.

Studies of the oxidation of leucine were complicated by the fact that under all experimental conditions tested a complete oxidation of the leucine could not be effected, even when concentrations of the substrate were reduced to 0.01 μM/ml. under conditions of vigorous aeration. This was in contrast to a wide variety of other substrates tested, such as glucose, acetate, succinate, ethanol, acetoacetate, etc., and suggests that the process of oxidative leucine degradation involved was a complex rather than a simple one perhaps involving a cleavage of the molecule to smaller ($C_2$ or $C_3$?) fragments which were then metabolized by various pathways. We were unable, therefore, to determine the value of the respiratory quotient (R. Q.), partly because of this fact, partly because of the variability of $CO_2$ evolution on leucine. It was unfortunate that the role of $CO_2$ in leucine metabolism could not be assessed in this manner since a comparison of oxidation rates in the presence and absence of $CO_2$ showed significant differences. Carbon dioxide could be involved in two ways. In the case of a mammalian pathway of leucine degradation, a refixation of evolved $CO_2$ would stimulate formation of end products and thus encourage the complete utilization of the supply of leucine; absorption of $CO_2$ by alkali would thus tend to inhibit leucine utilization. On the other hand, should leucine be degraded to fragments which were then metabolized <u>via</u> the TCA cycle,

accumulation of $CO_2$ in the test system would tend to inhibit leucine oxidation, and conversely, its removal would tend to shift the equilibria of reactions toward complete utilization. The data indicate that accumulation of $CO_2$ did in fact inhibit leucine oxidation.

In addition to the difficulties of $CO_2$ measurement already discussed, another practical difficulty encountered warrants mention. The indirect method of $O_2$-$CO_2$ estimation depends upon the use of flasks of different gas volumes, $V_g$, employing equal fluid volumes, $V_f$, or conversely, equal gas volumes with different volumes of fluid (91, p. 28). In the latter case with B. leucinophagum inconsistent results were obtained unless the extra fluid volume was distributed between the center well and another side arm of the flask not employed in the experiment. In other words, dilution of the reaction mixtures significantly affected gas exchange, i.e. the course of the reactions. This supports the hypothesis that $CO_2$ is involved in the reaction sequence, since the dilution of a reactant will shift equilibrium or even in some cases change the course of a reaction. These results suggest also that permeability effects play an important role in the metabolism of this organism.

The large amounts of $C^{14}O_2$ evolved from leucine-2-$C^{14}$ were completely unexpected. This observation indicates that a cleavage of the leucine molecule occurred, and suggests the involvement of a $C_2$ fragment. Once the isotopic carbon is separated, however, the remainder of the carbon skeleton

cannot be traced. It is significant, moreover, that an examination of the radioactive products of leucine-2-$C^{14}$ degradation reveals the same end products as formed from uniformly labelled leucine, except of lower specific activity as indicated by the lesser density of the spots on the radioautograms. This fact strongly supports the hypothesis of a degradation involving $C_2$ fragments, and, coupled with the observation that anaerobiosis completely inhibits leucine degradation, as it does the TCA cycle, would suggest that such $C_2$ fragments are metabolized via this route. The fact that no radioactive intermediates of the TCA cycle were isolated does not contradict this hypothesis since it is logical to expect that such intermediates would be far too reactive to accumulate. The refixation of evolved $C^{14}O_2$, whose presence can thus be explained as a result of TCA cycle decarboxylations, would lead to the isotopic labelling a large number of compounds not directly connected to the degradation of leucine.

When one considers the products of leucine degradation by B. leucinophagum (cf. Table 10), one striking fact is readily apparent: All of the end products which have been identified conclusively (with the exception of carbon dioxide) are amino acids which have previously been shown by other workers to be synthesized by microorganisms from $C_2$ or $C_3$ precursors which are related to pyruvate!

Aspartic and glutamic acids are formed by the well known reactions involving amination (or ammoniation) of the keto analogs, oxalacetate and α-ketoglutarate, which arise as intermediates in the TCA cycle (38, 55, 81, 49, 70, 50, 22, 58, 30, 94, 31, 32). Aspartic acid can also be formed by the action of aspartase, which catalyzes the reversible amination of fumarate, another TCA cycle intermediate (63, 96, 41, 36). In yeast aspartic acid is phosphorylated in the presence of ATP and $Mg^{++}$ to form L-β-aspartylphosphate (14), which is then reduced by a TPN-linked enzyme to the β-semialdehyde (15). This compound is reduced further in a DPN-dependent reaction to homoserine (16). These reactions have also been found to occur in Escherichia coli (95). The conversion of homoserine to threonine in E. coli requires ATP and pyridoxal phosphate, and may involve the formation of an intermediate compound (26, 25). Radioactive homoserine has not been detected in our experiments. The carbon skeleton of threonine (as α-ketobutyric acid) is involved in the condensation with a $C_2$ intermediate, derived from pyruvate, in the formation of α-keto-β-methylvaleric acid, the keto analog of isoleucine (90, 47, 5, 83).

Valine can be formed more directly from pyruvate by a condensation of pyruvate and a $C_2$ intermediate derived from pyruvate (57, p. 296).

Both cysteic acid and cystine may be formed enzymatically from cysteine. Although some cysteic acid was formed spontaneously during chromatographic analysis of our experiments, the concentrations detected would suggest its synthesis enzymatically also. This was later confirmed. Cysteic acid formation by means of a reversible dehydrogenase system has also been demonstrated in other organisms (71, 51), probably with the intermediate formation of cysteinesulfenic acid. This compound has never been isolated; it is probable that it undergoes a spontaneous dismutation to form cysteine and cysteinesulfinic acid, which has been observed to be oxidized to cysteic acid. The DPN-dependent oxidation of cysteine to cystine has been observed in yeast and higher plants (61, 67).

Cysteine may be formed in two ways: by the cysteine desulfhydrase reaction of pyruvate, $NH_3$ and $H_2S$, which has been shown to be reversible (73), and by the cystathionine pathway. The latter pathway has been demonstrated both in E. coli (48, 19, 29, 46) and in the mold, Neurospora crassa (88, 39, 43). Cystathionine is formed by the condensation of serine, derived directly from pyruvate (99) and also via $\beta$-hydroxypyruvate (45, 12), and homocysteine, derived from methionine in transmethylation reactions. Cystathionine thus formed is cleaved specifically to yield cysteine and

homoserine. The homoserine formed by this mechanism would not be labelled unless labelled methionine were involved.

Taurine, which has been identified in one series of experiments, has been shown to be formed from cysteinesulfinic acid (92), and also by the decarboxylation of cysteic acid (17). On the basis of these facts the metabolic scheme shown in Fig. 10 has been proposed.

The distribution of radioactivity in the products identified chromatographically from the degradation of $C^{14}$-leucine in time series experiments is in accord with this proposed scheme of reactions.

The radioactive products from the degradation of $C^{14}$-leucine which we have identified (Table 10) could also be formed from $C_2$ fragments derived from leucine by a pathway such as that found in pig heart and rat liver. These $C_2$ fragments (acetyl-CoA) are known to be formed from the $\alpha$- and $\beta$-carbons of leucine, split off in a manner analogous to the oxidation of fatty acids, and by the cleavage of acetoacetate in the presence of CoA. The fact that we have been unable to demonstrate these reactions in our bacterial system, however, suggests that a pathway differing from that found in animals may be implicated in the degradation of leucine by this species.

The mammalian pathway of leucine degradation is characterized by its dependence on the formation of CoA esters,

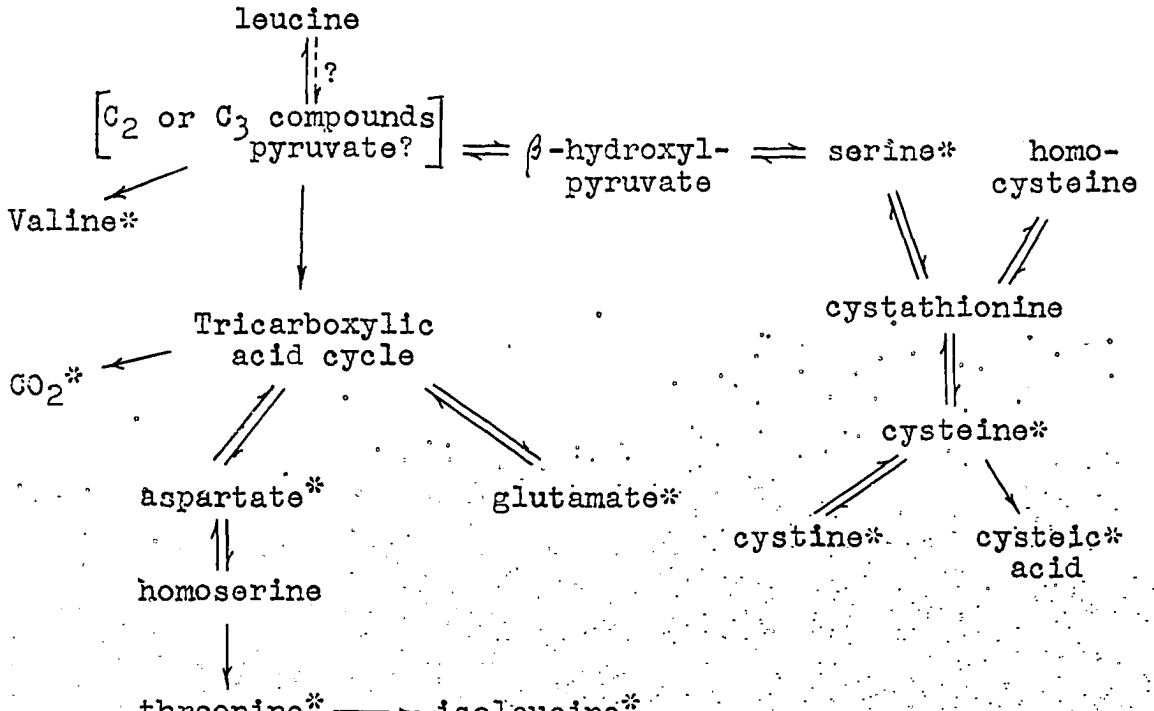


Fig. 10. Postulated metabolism of leucine by B. leucinophagum

The compounds marked (*) were identified during the degradation of $C^{14}$-leucine.

the fixation of $CO_2$ into HIV-CoA, and the accumulation of acetoacetate. These reactions take place under aerobic conditions and require the presence of thiols (10, 11, 27).

The fixation of $CO_2$ in the presence of leucine by B. leucinophagum is strongly inhibited by molecular oxygen, even though reducing substances (thiols) are present in the reaction mixture. This low fixation might be explained by a partial loss of enzyme activity in our preparations which would cause the supply of HIV-CoA, the actual substrate of mammalian $CO_2$ fixation, to be limiting. However, although an anaerobic fixation of $CO_2$ does occur, it is not dependent upon CoA or the presence of thiols, as is the mammalian reaction. (Reduced glutathione was substituted for cysteine with essentially the same results.) Furthermore, neither HMG-CoA nor acetoacetate could be detected as a result of this $CO_2$ fixation. It is not likely that the endogenous concentration of CoA in the crude extracts employed could satisfy the requirements of a CoA-dependent sequence of reactions under the conditions of our experiments (25 to 50 µM leucine). Moreover, CoA esters could not be detected chromatographically in reaction mixtures of leucine and CoA with either whole cells, acetone powders or cell juices.

The data of Table 15 indicate that acetate, rather than acetate plus acetoacetate, is more directly involved in the fixation of $CO_2$. Furthermore, if the same reversible

$CO_2$ fixation reactions were taking place in our enzyme preparation as in mammalian tissues and yeast, one would expect that some radioactive acetoacetate would be formed by exchange which could be trapped as the 2,4-dinitrophenylhydrazone (the derivative is more stable that the parent compound). No evidence of $C^{14}$-acetoacetate formation could be found, however. To determine whether HMG-CoA might have been formed even though the HIV-CoA-$CO_2$ fixation system might have been impaired during the preparation of our extract, malate (100 μM), shown to inhibit the cleavage of HMG-CoA (11), was added after the reaction had been allowed to proceed for 30 min. No chromatographic evidence for the formation of HMG-CoA was obtained. The products of the $CO_2$ fixation were not further identified.

It is interesting to note that most of the degradation products of $C^{14}$-leucine by whole cells of B. leucinophagum are amino acids, which are then incorporated into peptide or protein under the conditions of our experiments. This is in contrast to the degradation of leucine by S. cerevisiae, during which the carbon skeleton is not appreciably utilized to synthesize other amino acids (77).

The biosynthesis of leucine has been studied in a number of microorganisms (E. coli, N. crassa, S. cerevisiae and Torula utilis) by the use of mutant strains and direct and indirect isotope methods (64, 86, 2, 3). Though all of

the reactions have not been demonstrated, the six carbon atoms of leucine are known to arise directly or indirectly from pyruvate. Other carbon sources, such as glucose, lactate and acetate, are first converted to pyruvate before they are utilized. A bacterial degradation of leucine which was essentially the reverse of the biosynthetic pathway could thus furnish a supply of $C_2$ or $C_3$ intermediates, related to pyruvate, which could be metabolized via the TCA cycle and serve as precursors of the end products we have identified. All the necessary enzymes for leucine biosynthesis appear to be present in our test organism, since we have shown (47) that B. leucinophagum grows readily in Koser's medium with citrate as sole carbon source, $NH_4^+$ as sole nitrogen source.

# SUMMARY AND CONCLUSIONS

1. A rod-shaped, Gram positive bacterium, isolated from soil on L-leucine as sole source of carbon and nitrogen, was found to possess properties typical of the genus Brevibacterium Breed, family Brevibacteriaceae Breed. It appears to represent a new species for which the name Brevibacterium leucinophagum has been proposed.

2. The cultural, morphological and physiological characters of the organism were determined and are described.

3. Manometric studies using washed whole cells of the test organism showed large amounts of oxygen to be taken up without initial lag periods on L-leucine. D-leucine was not metabolized, and caused some inhibition of the oxidation of the L-isomer.

4. Carbon dioxide was evolved from L-leucine by washed whole cells, but variations due to undetermined factors prevented the determination of the respiratory quotient. The presence of $CO_2$ was shown to influence rates of leucine oxidation.

5. No accumulation of free $NH_3$ was detected in the presence of whole cells, but $NH_3$ was detected with acetone powder preparations, suggesting the presence of a leucine deaminase.

6. The presence of a very active leucine-$\alpha$-ketoglutarate transaminase was noted in whole cells, acetone-dried cells and crude sonic extracts. A carbonyl compound which appeared to be $\alpha$-ketoisocaproate accumulated when sonic extracts and acetone powders were used.

7. When DL-leucine-2-$C^{14}$ was degraded by washed whole cells under aerobic conditions, large amounts of $C^{14}O_2$ were evolved, suggesting a pathway involving $C_2$ fragments. A number of radioactive amino acids were detected as products of the degradation.

8. No degradation of leucine-2-$C^{14}$ occurred under anaerobic conditions.

9. When uniformly labelled $C^{14}$-L-leucine was degraded aerobically by whole cells, the same radioactive end products were detected, except with higher specific activities.

10. The radioactive products were identified chromatographically as amino acids which were readily incorporated into peptide or protein. Two ninhydrin-negative spots, one formed in fairly high concentrations, were unable to be identified.

11. The distribution of radioactivity from degraded $C^{14}$-leucine was found to vary during the course of time series experiments over the period 15 to 75 min.

12. Attempts to prepare enzymes in a cell-free state active for complete leucine degradation were unsuccessful.

13. All attempts to demonstrate reactions known to occur in pig heart and rat liver during leucine degradation were negative. No acetoacetate from leucine accumulated, no CoA ester formation could be detected, no fixation of $CO_2$ to form $\beta$-hydroxy-$\beta$-metylglutaryl-CoA occurred, and no exchange of $C^{14}O_2$ with acetate plus acetoacetate took place. On this basis it is concluded that leucine is degraded by whole cells of B. leucinophagum by a sequence of reactions different from that found in mammalian tissues.

14. The presence and operation of a tricarboxylic acid cycle is concluded. This is based on the observations that the test organism grows readily in the presence of $NH_4^+$ and various members of the TCA cycle as sole sources of carbon, as well as on glutamate, aspartmate and alanine; oxidative activity of the organism is stimulated by TCA cycle compounds and close relatives; these reactions are sensitive to compounds known to inhibit TCA cycle reactions; the interconversion and accumulation of TCA cycle intermediates can be detected chromatographically in the presence of TCA cycle inhibitors.

15. Evidence for the adaptive synthesis of a malonate-metabolizing enzyme system was obtained.

16. Evidence for the presence of cysteine desulfhydrase was obtained with whole cells anaerobically and with acetone-dried cells. The presence of a cysteine dehydrogenase (for

the formation of cystine) and a "cysteine oxidase" (for the formation of cysteic acid) was indicated.

17. On the basis of this evidence, a pathway of leucine degradation by whole cells of B. leucinophagum differing from that found in mammalian tissues is postulated. The evidence for the formation of $C_2$ or $C_3$ fragments related to pyruvate, which could explain the formation of the end products identified, by a pathway essentially the reverse of the biosynthetic route in microorganisms is discussed.

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